STUDIES ON BASIC MECHANISMS OF ACUTE INFLAMMATION: THE RELEASE OF LYSOSOMAL ENZYMES FROM HUMAN LEUKOCYTES DURING PHAGOCYTOSIS

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by

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CONTENTS:

I. INTRODUCTION

The discoveries made at the end of the 19th centruy concerning the importance of infectious micro-organisms in human disease provoked great interest in the physiologic response of the body to infection. It was at this time that the biology of inflammation was first carefully studied and accurately described. In 1882 Julius Cohnheim presented his lectures describing the microscopic vascular events which occur in acutely inflammed tissues as he observed them in the mesentery and tongue of the frog; from his descriptions -- of vascular dilatation, transudation of fluid, margination of white cells against vessel walls and migration of the white cells into the extravascular spaces -- have sprung several channels of basic research on mechanisms of acute inflammation.[1]

Soon after Cohnheim's reports, Elie Metchnikoff clarified the role of the inflammatory process and particularly of polymorphonuclear leukocytes (neutrophils) in natural protection against inflammatory disease. "The diapedesis of the white corpuscles, their migration through the vessel wall into the cavities and tissues, is one of the principal means of defence possessed by an animal. As soon as the infective agents have penetrated into the body, a whole army of white corpuscles proceed towards the menaced spot, there entering into a struggle with the micro-organisms."[2]

Metchnikoff represented the tissue damage of acute

inflammation largely through military metaphors -- the necessary result of battles between the invading microbe and host. However, his studies recognized the importance of digestive "ferments" and "cytases" contained in neutrophils for the function of these cells. "The leukocytes, having arrived at the spot where the intruders are found, seize them after the manner of Amoebae and within their bodies subject them to intracellular digestion. This digestion takes place in the vacuoles in which usually is a weakly acid fluid which contains digestive ferments." [3]

Since the turn of the century, the "ferments" and the process of phagocytosis have been studied with more refinement by techniques of modern cell biology and enzymology. In particular, the digestive enzymes contained in neutrophils have been studied as they act as mediators of the tissue damage in acute inflammation. The release of these digestive enzymes during phagocytosis is the subject of several studies with human neutrophils in vitro to be presented in this report.

It had been proposed at the time of Metchnikoff's studies that oneof the functions of inflammatory leukocytes might be to deliver bactericidal substances to the sites of infection, for it was known that white cell extracts lent bactericidal properties to normal serum. Metchnikoff's observations, however, led him to believe that the neutrophils' bactericidal "ferments" served essentially the mechanisms of phagocytosis and intracellular digestion of foreign particles or microbes and were released

into the tissues only with the disruption of these cells. "The cytases must be grouped with the soluble ferments which are not thrown off by the phagocytes as long as these remain intact. Immediately these cells are injured, however, they allow a part of their cytases to escape." [4]

Interest in the character and function of the proteolytic and other hydrolytic enzymes contained in neutrophils has focused not only on the role of these enzymes in the protection against infecting micro-organisms but also on their role in processes that are themselves harmful to the body. As soon as the protective function of inflammation was recognized, it was also recognized that the inflammatory process could be misdirected and could itself produce disease.

Experimentation with models of inflammation wherein the inflammatory response rather than the inflammatory stimulus is harmful has accompanied the increasing study and recognition of diseases associated with diffuse or localized inflammation which offers no apparent protective function. Among these diseases are the so-called "immune complex" vasculitides, post-streptococcal glomerulonephritis, and acute gouty arthritis.

The Arthus Phenomenon, in particular, first described in 1903, has been an experimental archetype of misdirected inflammation.[5] Arthus observed that once an experimental animal is immunized against a foreign protein, this protein injected into the skin of the animal provokes an inflammatory response at the site of injection resulting in tissue necrosis. It was

demonstrated subsequently that this tissue damage is a direct consequence, not of the antigen-antibody complexes which form in the skin of the experimental animal, but of the classic events of inflammation which are provoked by these protein complexes.[6,7] Neutrophils are attracted to the sites where deposition of antigen-antibody complexes occurs, and there they phagocytize these complexes.[6,7,8] The tissue damage resulting from the experimental inflammation in the Arthus Phenomenon depends upon the presence of neutrophils in the tissues. Tissue damage does not occur when neutrophils are prevented from accumulating either by rendering the animal leukopenic [9,10,11] or by inhibiting reactions in the blood complement system, initiated by the formation of antigen-antibody complexes, which attract neutrophils to the experimental site.[12,13]

A model of misdirected inflammation which has been explored similarly is one in which urate crystals are infused into the joints of humans or animals; the crystals provoke an acute inflammation in which the inflammatory exudate consists primarily of neutrophils, which may be observed to phagocytize the crystals.[14,15] Infusion of urate crystals into leukopenic animals results neither in inflammation nor in tissue injury.[16]

Investigations concerning the mechanisms of tissue injury produced by phagocytizing neutrophils in these experimental models have commanded particular interest in recent years, for an understanding of these mechanisms promises the eventual control of various inflammatory diseases. One of the

first proposals which related tissue injury to the accumulation of neutrophils in the inflammatory response was that the concentrations of lactic acid produced by accumulated neutrophils are toxic to tissues.[17,18] It has been well demonstrated that an increased production of lactic acid by neutrophils occurs during phagocytosis and under anaerobic conditions; also, direct measurements of pH in inflammatory, transudative fluids in which neutrophil accumulation occurs have shown a fall in pH over time as a function of the number of neutrophils present.[19,20,21] More recently, however, attention has turned to the various digestive enzymes contained in neutrophils as important mediators of tissue injury in acute inflammation.

By the mid-1950's it was known that neutrophils contain enzymes capable of degrading polymers of amino-acids, carbohydrates, lipids, and phosphorylated or sulphated compounds and are thus well equipped for the digestion of a variety of tissue substrates.[22,23] A significant advance in understanding the mobilization of these enzymes was made when it was recognized that the enzymes are stored in intracellular organelles long characterized morphologically as "granules." In 1955 De Duve described in rat liver cells a class of cytoplasmic organelles, "lysosomes," which contain several distinct acid hydrolytic enzymes; these enzymes were found to exhibit activity only when released from the organelles into a soluble phase.[24,25,26] It was recognized that enzymes contained in neutrophil granules

behave after the manner of lysosomal enzymes in that they exhibit activity only when the intact granules are disrupted.[27] Morphological studies of phagocytizing neutrophils, with both light and electron microscopy, have elucidated the mechanism by which their "lysosomal" enzymes are mobilized for the digestion of phagocytized particles; the membrane shell of the granules fuses with the membrane enclosing the phagocytic vacuole containing an ingested particle, and therby the granules release their enzymes into the vacuoles. In this way the neutrophil is "degranulated" during phagocytosis.[28-32]

Various enzymes isolated from neutrophil granules have been shown to be active in degrading tissue constituents; collagenase, neutral protease, elastase, and acid cathepsins have been variously described, active individually or together against collagen, protein polysaccharides, elastin, arterial cell walls, and isolated vascular basement membrane.[33(review)] These enzymes have been identified in neutrophil granules together with various acid hydrolases present in lysosomes of other cell types[27]; with certain enzymes or proteins that have specific anti-bacterial activity (Lysozyme,"Leukin," and "Phagocytin")[8,27]; and with a group of "basic cationic proteins" which together with bactericidal actions cause increased vascular permiability.[8,34-38,38a]

Although mechanisms have been shown for the mobilization of these various substances in the intracellular digestion of phagocytized particles, mechanisms for the release of these granular substances extra-cellularly have been less clear.

There is strong circumstantial evidence, however, that lysosomal enzymes and proteins are released from neutrophils and play an important part in the tissue damage of acute inflammation. Evidnece for this already mentioned includes: 1) experimental models of acute inflammation in which neutrophils must be present in tissues for tissue injury to occur, 2) the activity of neutrophil granular enzymes in degrading various tissue constituents. Evidence that neutrophil granular enzymes mediate tissue injury also includes: 3) the experimental production of tissue damage by direct infusion of neutrophil granule suspensions or granule extracts into the skin of animals,[8,35,36,39] 4) the inhibition by certain anti-inflammatory drugs of experimentally induced diruption of isolated lysosomes in vitro, or inhibition of neutrophil degranulation during phagocytosis.[8,33,40,41]

It will be remembered that Metchnikoff in his original reports argued against the proposition that "cytases" escape from neutrophils during their normal function. Since 1960, however, several studies with human and non-human neutrophils in vitro have shown that a portion of their granular enzymes are extruded when these cells phagocytize various paricles -- bacteria, immune complexes, yeast and starch granules.[33,42-52]

Studies to be presented in this report provide direct evidence that neutrophil granular enzymes not only disappear from these cells during phagocytosis but also appear in the surrounding media in which the leukocytes are phagocytizing particles. Also these studies demonstrate that the immediate fate

of granular enzymes during phagocytosis depends in part upon the respective stabilities of these enzymes in soluble phase. Furthermore, these studies demonstrate that the release of granular enzymes from phagocytizing leukocytes may be inhibited by certain anti-inflammatory drugs (colchicine and cortisol) but not by another such drug (salicylate).

II. THE RELEASE OF LYSOZYME FROM HUMAN LEUKOCYTES DURING PHAGOCYTOSIS

Experiments with rabbit neutrophils *in vitro* have demonstrated that various granule-bound enzymes are redistributed within the cells as a consequence of phagocytosis of bacteria; these enzymes were reported to be released from the granule fraction of the cells into the cytoplasm or soluble fraction of the cells.[42] In these studies only small amounts of granular enzyme activities were recovered from the extra-cellular medium of the phagocytizing cells, and no decrease of net intracellular enzyme activities was found to occur.[42] Subsequent studies with human leukocytes *in vitro* were reported to demonstrate a comparable redistribution of granular enzymes intracellularly when these cells phagocytized immune complexes (Gamma G - Rheumatoid Factor); similarly these studies did not report significant losses of enzyme activities from the leukocytes.[44]

More recently, however, several studies have been reported which demonstrate or suggest significant release of granule associated enzymes from neutrophils consequent to phagocytosis of a variety of particles *in vitro*. Both human[45,46,48,49,51] and rabbit[47,50] neutrophils have been used in these experiments; enzymes shown to be released into the extra-cellular media of phagocytizing cells include: lysozyme,[45,49,50] acid phosphatase,[47,48] acid protease (cathepsin),[47,49] B-glucuronidase,[46,48,51] alkaline phosphatase,[48] and peroxidase.[48] Release of granular enzymes

has been reported to occur with phagocytosis of bacteria (E. coli,[47,48] Bacillus subtilis,[47] M. lysodeikticus,[50] and Staphylococci[49]), immune complexes (bovine serum albumin-anti BSA,[50] precipitates of Staphylococcus cell-wall antigens in specific immune sera[45]), and inert particles (globulin coated latex beads,[47] zymozan particles,[47,51] and starch granules[46,48]).

In the following experiments I have investigated the intracellular redistribution and the extra-cellular release of lysozyme from human leukocytes during phagocytosis of Staphylococcus.

Methods

Glassware:

All glassware used in these experiments was made sterile and pyrogen free by heating st 160°C for 2 hrs. In addition, glassware used in the isolation and incubation of leukocytes (pipettes, centrifuge tubes, and flasks) was siliconized to prevent sticking of the cells to the glass.

Isolation of Leukocytes from Human Blood:[40]

Up to 150 ml of blood was drawn from the antecubital veins of normal volunteers; the blood was heparinized (10 mg/100 ml), mixed with 2 vol. of Dextran solution* and allowed to sediment in 100 ml cylinders at room temperature for 20-30 min. The leukocyte rich supernatant was then centrifuged in 50 ml tubes at 1200 rpm (800 g) in an International Centrifuge, model PR-2, for 15 min. The new supernatant was removed and the cell button re-

*3% Dextran of M.W. 100,000-200,000 (Nutritional Biochemical Corps., Cleveland, Ohio) in normal saline, autoclaved for 2 hrs and stored at 4°C.

suspended in modified Krebs-Ringer phosphate buffer at pH 7.4.* The resulting cell suspensions were again centrifuged and the supernatants removed. The cell buttons were each gently mixed over ice with 3 ml sterile distilled water for 20 sec, to lyse the red cells, and then mixed with 1 ml 3.6% NaCl to restore isotonicity. The cells were washed once more in buffer, resuspended in a small volume of buffer, counted with a Coulter particle counter, and adjusted to the desired concentration. Wright-stained slides were prepared for differential leukocyte counts of all cell isolates; it was found that regularly 60-75% of the leukocytes were neutrophils.

Heat-killed Bacteria:

Cultures of Staphylococcus** albus were stored on blood agar plates at $4^{o}C$ and passed to new plates every three weeks. For the experiments, the bacteria were cultured for 18 hrs in beef-heart infusion broth, centrifuged, washed once in normal saline, and then suspended in a small volume of saline. The concentration of bacteria was estimated from a constructed curve of transmittance at 600 mu and confirmed by quantitative plating in agar. The bacteria were killed by autoclaving at 15 lbs pressure for 20 min. Aliqots of 4-5 ml were frozen and stored at $-20^{o}C$.

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*this buffer was sterilized by autoclaving for 2 hrs the individual constituent solutions which were: Krebs-Ringer base (100 parts .9% NaCl, 4 parts 1.15% KCl, 0.6 parts 1.22% $CaCl_2$, 0.2 parts 3.82% $MgSO_4$) and phosphate buffer (14.3 gm Na_2HPO_4 in distilled (cont.)

**a strain originally obtained from a finger abscess

Incubation of Leukocytes:

1.0 ml of leukocyte suspension (containing 2.6-3.4 x 10^7 leukocytes/ml) were incubated in 25 ml flasks with 1.5 ml buffer, 0.3 ml autologous serum from the blood donor, and 0.2 ml saline or bacteria suspension, for 1 hr at 37.5°C in a shaking water bath. 25 ml Erlenmeyer flasks with 2.7 ml buffer and 0.3 ml serum were also prepared and incubated concurrently. In each experiment flasks containing leukocytes with or without bacteria were incubated in duplicate; in each experiment phagocytizing leukocytes and control leukocytes without bacteria were prepared at the same time from the same blood donor and were incubated concurrently.

Preparation of Leukocytes for Morphology:[29,40]

Drops of cell suspensions after incubation were placed on sterile slides, then covered with sterile coverslips. After incubating the slides for 20 min at 37°C in covered petri dishes containing a moist filter paper, the coverslips were removed, quickly air dried, then stained with Wright's stain.

Preparation of Cell Fractions:

After incubation, leukocyte suspensions were centrifuged in 12 ml tubes at 1200 rpm (International Centrifuge) for 10 min at 4°C. The supernatants represented the "extra-cellular

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water, adjusted to pH 7.4 with 16(±) ml of 1 N HCl, and made up to 1000 ml.) For each experiment 10 mg/100 ml heparin was added to the final K-R buffer solution which consisted of 105 parts Krebs-Ringer base and 20 parts Phosphate buffer.

media fractions." The cell buttons were either resuspended in 0.2 M sucrose up to 3.0 ml, representing the "whole cell fractions," or they were resuspended for preparation of "nuclear," "granular," or "soluble" cell fractions. The preparation of these latter three cell fractions was done according to a previously reported method.[53] By this method the cell buttons, having been separated from the media, were resuspended in 5.5 ml of 0.2 M sucrose; 0.3 ml heparin (5000 U/ml) was added to the cell suspensions and gently mixed with a pasteur pipette until marked viscosity appeared, indicating cell lysis. The lysate suspensions were then centrifuged at 1200 rpm (International Centrifuge) for 10 min at 4°C†; the supernatants were transferred to high speed Sorvall tubes and centrifuged at 10,000 rpm (12,000 g, Sorvall Centrifuge Superspeed SS-3) for 20 min, while the buttons were resuspended to 3 ml in 0.2 M sucrose and saved as the "granular" fractions. All fractions were frozen and stored at -20°C not more than three days until enzyme determinations could be made. When enzyme activities were measured, the values obtained for the "soluble" fractions were multiplied by a factor of 2 in order to adjust for differences in dilution.

Determination of Lysozyme:

The assay for lysozyme activity used in these experiments is an adaptation of a commonly used turbidometric method.[54] 3.0 ml aliquots of substrate solution* were warmed to

*80 mg of a standard M. lysodeikticus cell wall preparation, Bacto-Lysozyme Substrate, Difco Laboratories, Detroit, Mich., (cont. next pg

†buttons from this centrifugation were resuspended to 3 ml in 0.2 M sucrose and saved as the "nuclear" fractions.

37°C. 0.5 ml of sample to be measured was then added to an aliquot of the substrate suspension, mixed, and transferred to a spectrophotometer cell* which had also been warmed to 37°C. Lysozyme activity, or lysis of the cell walls in suspension, was represented by change in optical density at 540 mu over time; enzyme activity was expressed in terms of ugms. of egg-white lysozyme** and in terms of ugms. of purified human lysozyme isolated from leukemics' urine,*** according to standard curves prepared at the time of the enzyme assays.† Before enzyme determinations were made, all fractions were freeze-thawed 5 times with 0.1 ml of 1.0% Triton-X 100, a neutral detergent.

Determination of Lactic Dehydrogenase:

Leukocyte LDH determination was measured by an accepted method for serm LDH determination.[55] 0.1 ml of the sample to be measured† was mixed with 2.85 ml of Pyruvate solution (0.05 M phosphate buffer at pH 7.5, 3.1×10^{-4} Pyruvate) and 0.05 ml DPNH solution (8×10^{-3} M B-DPNH in Pyruvate solution). Change in optical density at 340 mu, which occurred with oxidation of DPNH, was recorded with a spectrophotometer equipped with a recorder* ; temperature of the reaction was controlled at 25°C. LDH activity was expressed in Wroblewski units.[55]

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suspended in 50 ml of buffer at pH 6.2, Bacto-Lysozyme Buffer, Difco. Labs., and homogenized with a teflon pestle for 2 min.

*Hitachi Perkin-Elmer Spectrophotometer #139, equipped with a Sargent Recorder SRL

**Bact-Lysozyme, Difco Labs.

***Human Lysozyme, courtesy of Dr. Stuart Finch

****Rohm and Haas Co., Phil.,Pa.

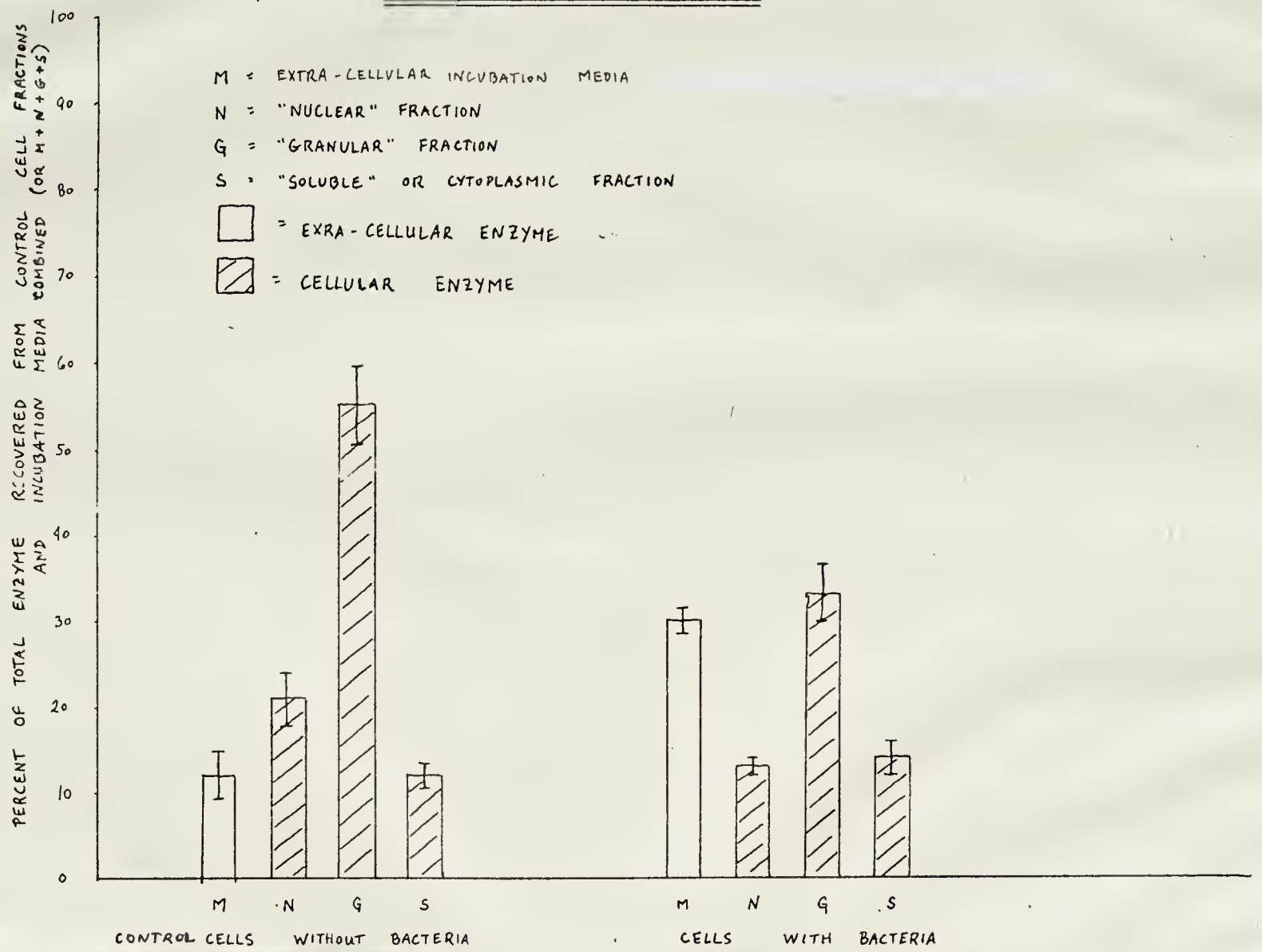
† measurements of lysozyme and LDH in the "media" fractions were always adjusted for enzyme activities in the serum which made up a portion of the incubation media.

Results

The localization of lysozyme activity in the extracellular media and in the various cell compartments, when leukocytes have been incubated for 1 hr with and without bacteria, is illustrated in Fig. 1. Active phagocytosis with most neutrophils ingesting more than 5 bacteria was demonstrable microscopically for the leukocyte cultures incubated with bacteria (at a multiplicity of 10-20 bacteria to 1 neutrophil). Only neutrophils and monocytes (the latter cell type representing no more than 5-8% of the isolated leukocytes) were observed to ingest bacteria. In Fig. 1 it is evident that the greatest changes in distribution of lysozyme which occur with phagocytosis are in the "granular" and in the "medium" fractions, indicating a release of lysozyme into the incubation media during phagocytosis. A negligible increase of lysozyme activity was observed in the "soluble" or cytoplasmic fraction -- a finding which differs from observations made with other granular enzymes in rabbit neutrophils.[42] This finding, however, agrees with observations of others with acid and alkaline phosphatase, B-glucuronidase, and peroxidase in human leukocytes.[46,48]

The decrease in lysozyme activity that occurs in the "nuclear" fraction with phagocytosis may be considered to represent for the most part a decrease in the granule bound fraction, for the "nuclear" fraction was found microscopically to contain a proportion of whole leukocytes which had not been lysed in the fractionation procedure, as well as a proportion of adherent

FIG. 1 DISTRIBUTION OF LYSOZYME IN LEUKOCYTES WITH AND WITHOUT PHAGOCYTOSIS



Total enzyme recovered* = 3.8 - 6.4 ugm (human lysozyme)/ml (10^7 leukocytes/ml), or 12.9 - 24.4 ugm (egg-white lysozyme)/ml(10^7 leukocytes/ml); the results represent mean values for 4 experiments; Standard Errors are indicated.

incubation with bacteria 60 min ; multiplicity 20 bacteria to 1 neutrophil

* total enzyme recovered from M, N, G, and S fractions of control cells without bacteria

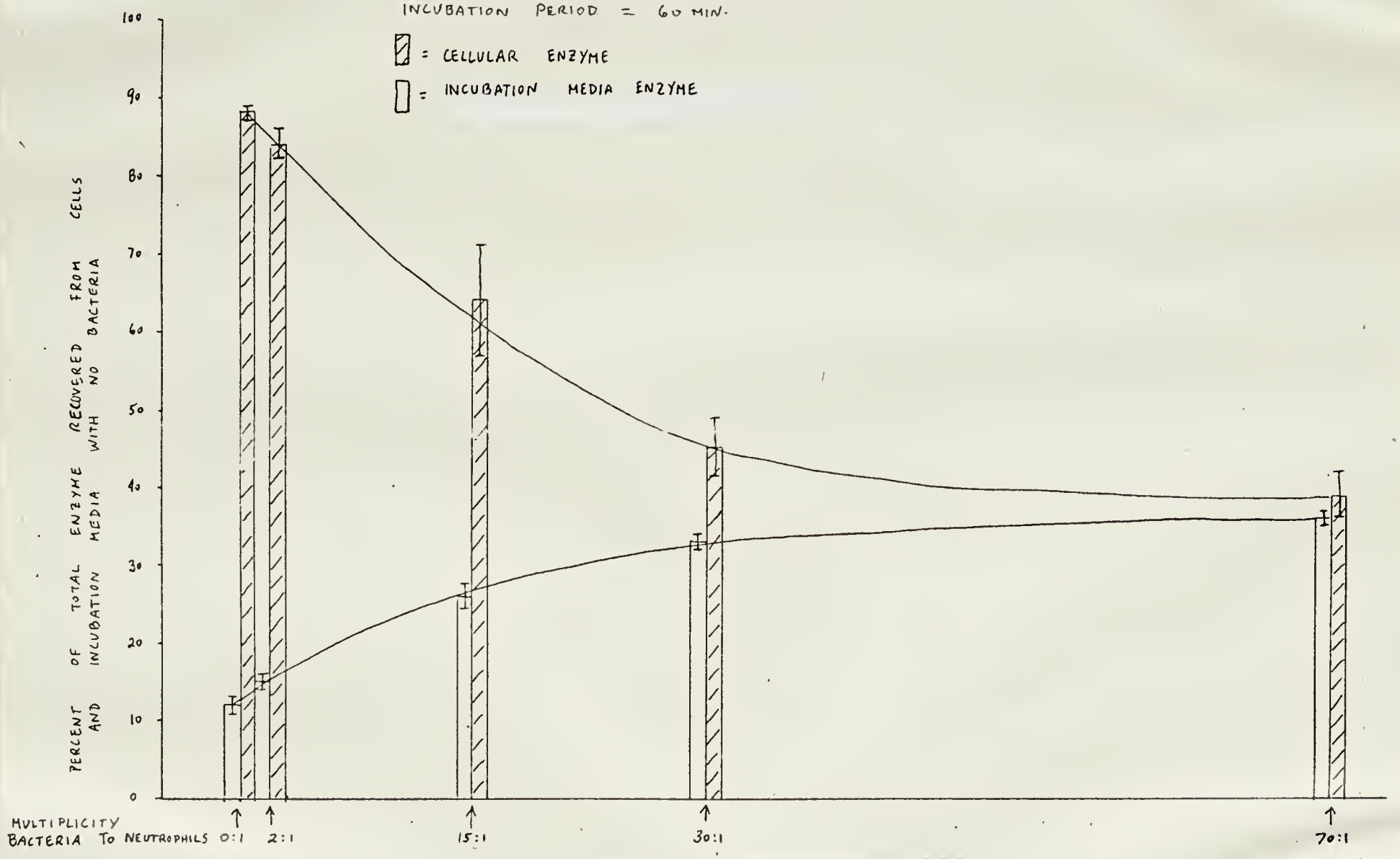
granules; this finding corresponds to the experience of others in preparing leukocyte fractions.[27,53]

In these experiments, as the media enzyme activity is found to increase after phagocytosis, the total cellular enzyme is found to decrease. Also of note is the fact that the total enzyme (cellular + media enzyme) recovered from the cultures in which phagocytosis occurs is less than that recovered from control cultures.†

The kinetics of the shift of lysozyme activity from the cells to the extra-cellular incubation media during phagocytosis were investigated with varying degrees of phagocytic challenge (multiplicity of bacteria/ neutrophil) and with varying incubation times. The results of these experiments are presented in Figs. 2 and 3. It is evident in Fig. 2 that the extra-cellular release of lysozyme increases with the phagocytic challenge up to a certain point (about 30 to 1, bacteria to neutrophils) at which multiplicity the release of enzyme appears to remain fairly constant; this finding is perhaps best explained by the supposition either that the leukocytes become saturated with ingested bacteria at a high multiplicity or that the mechanism by which enzyme is released from the cells has a limit. When the leukocytes are incubated at a high multiplicity (Fig. 3), it is evident that most of the extra-cellular release of lysozyme occurs during the first 30 min of incubation, by which time most of the phagocytosis is known to have occurred.[27] The data of Fig. 3

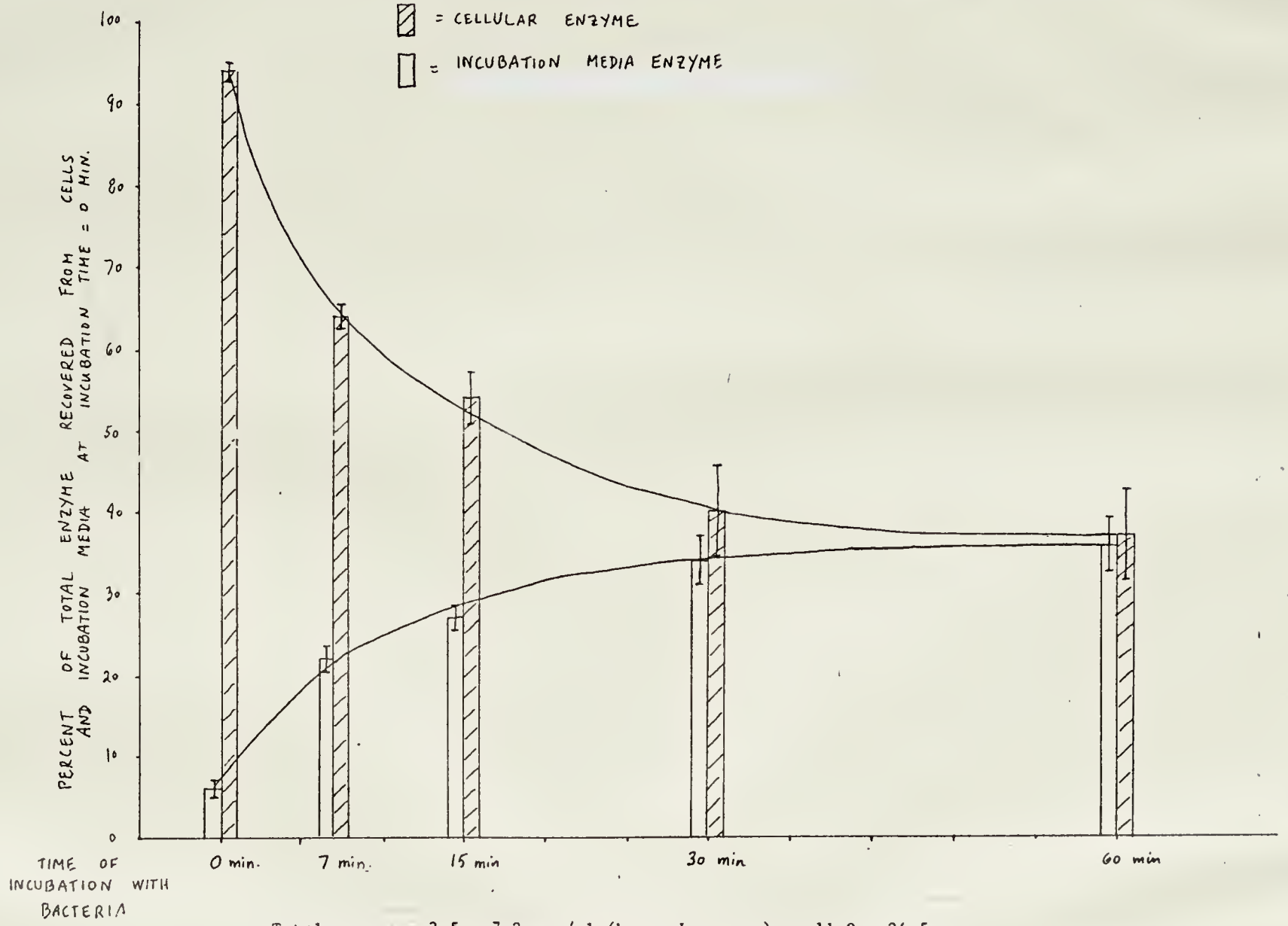
† in Fig. 1 total enzyme activity recovered from the M,N,C, and S fractions of phagocytizing cells is 90% of that recovered from combined fractions of control cells.

FIG. 2 EXTRA-CELLULAR RELEASE OF LYSOZYME WITH INCREASING PHAGOCYTIC CHALLENGE



Total enzyme= 4.1 - 8.2 ugm/ml (human Lysozyme) or 13.5 - 27.7 ugm/ml (egg-white Lysozyme); SEM's are indicated; the results represent mean values for 4 experiments.

FIG. 3 EXTRA - CELLULAR RELEASE OF LYSOZYME OVER TIME OF INCUBATION AT MULTIPLICITY : 30 - 90 BACTERIA to 1



Total enzyme = 3.5 - 7.2 ugm/ml (human Lysozyme) or 11.9 - 24.5 ugm/ml (egg-white Lysozyme); results represent mean values for 4 experiments; SEM's are indicated.

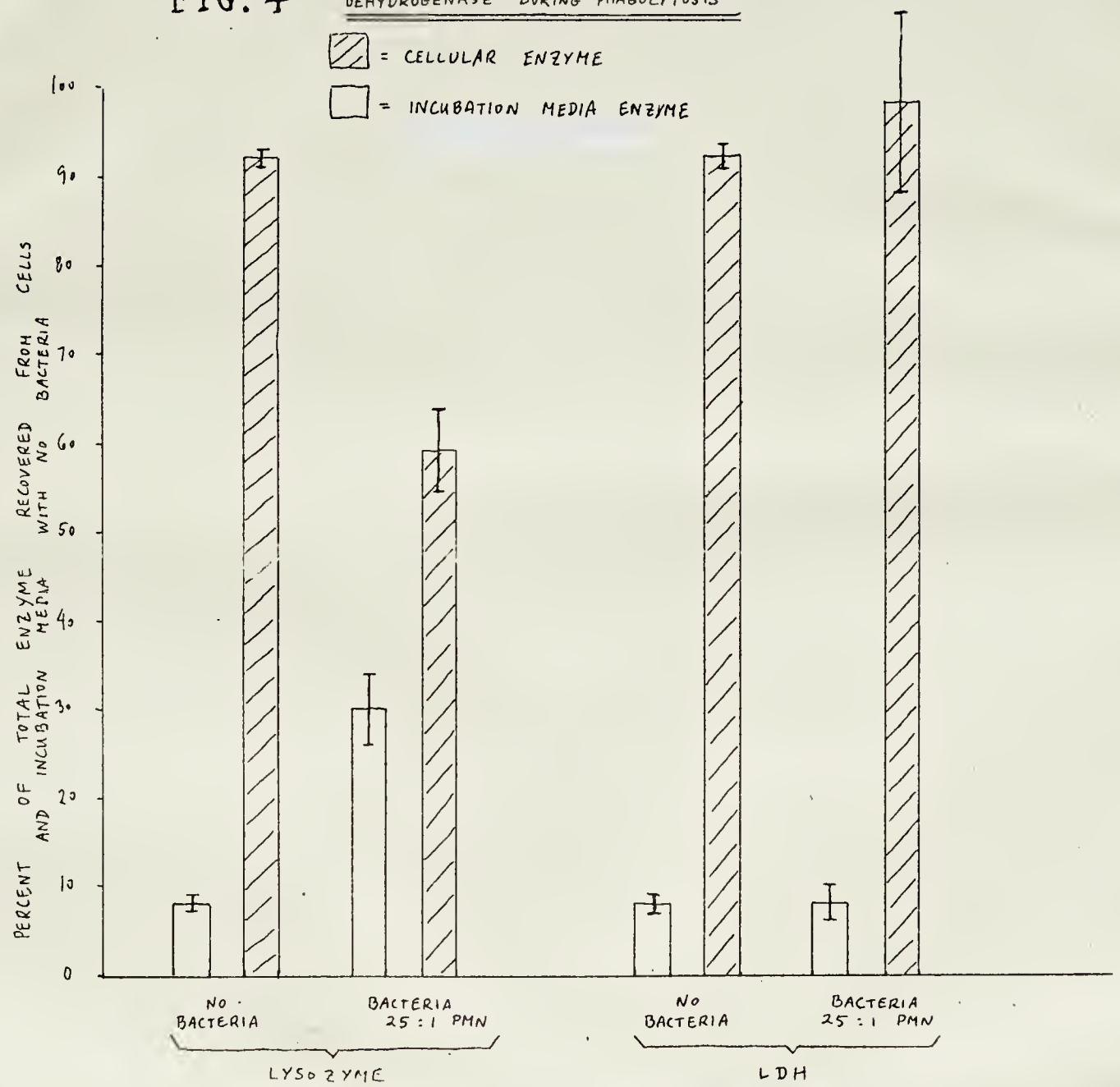
correspond to previously reported findings which related the appearance of granular enzyme extra-cellularly to time of phagocytosis.[49,50] The values for total leukocyte lysozyme, which were found to vary with different leukocyte isolations and with different blood donors, agree with absolute values of lysozyme activity recovered from leukocytes reported by others (12.0 - 22.0 ugms egg-white lysozyme/ 10^7 WBC's isolated from five normal controls).[56]

Since it was important to know if the extra-cellular release of lysozyme, which was observed to occur with phagocytosis, represents injury to neutrophils, phagocytosis experiemnts were performed in which both lysozyme and lactic dehydrogenase, a non-granule bound enzyme located in the cytoplasm, were measured concurrently. The results are presented in Fig. 4. These experiments demonstrate that, while lysozyme is released from the leukocytes, coincidental release of LDH does not occur. These results confirm findings of others who compared the fate of non-granular and granule bound enzymes in a similar experimental system.[52] These results also support the conclusion that mobilization of granular enzymes during phagocytosis is not accompanied by decreased viability of the leukocytes; studies with similar experimental systems have demonstrated, by dye exclusion techniques, no loss of viability of neutrophils as a consequence of phagocytosis.[49,51]

Discussion

It would be dangerous to assume that the release of

FIG. 4 REDISTRIBUTION OF LYSOZYME AND LACTIC DEHYDROGENASE DURING PHAGOCYTOSIS



Total Lysozyme = 4.3 - 5.2 ugm/ml (human Lysozyme), or 14.6 - 17.7 ugm/ml (egg-white Lysozyme); Total LDH = 540 - 710 units/ml; Results represent mean values of 4 experiments, with SEM's indicated; Differences between Lysozyme and LDH media and cell enzyme are significant, $p < 0.02$ (by paired t-test)

incubation time = 60 min.

lysozyme with phagocytosis is a model for the behavior of other enzymes associated with neutrophil granules. In fact, there is evidence that lysozyme may be differentiated from other granular enzymes, at least in rabbit neutrophils, according to the extent to which it is associated with the granular fraction (about 50%, compared with 60-70% for other enzymes, eg. acid phosphatase and B-glucuronidase),[27] and according to the specific granules with which lysozyme is associated.[57,58] In studies of rabbit neutrophil granules, investigated by density gradient centrifugation, lysozyme activity was found to be associated with two types of granules -- small sized granules where most other granular enzymes are located, and somewhat larger granules in which alkaline phosphatase was also found.[57,58] These studies suggest that human neutrophils, also, may contain divers granule types. Therefore, one must be cautious in drawing conclusions about neutrophil granular enzymes in general from studies of lysozyme alone.

On the other hand, the results of these experiments with human leukocytes, demonstrating the shift of lysozyme activity from the granules to the extra-cellular media, correspond very closely to previously reported experiments that demonstrate a shift of B-glucuronidase from the "granular" to the extra-cellular fractions when human leukocytes phagocytize starch particles.[46] In another study the peroxidase of human neutrophils was found to behave similarly with phagocytosis.[48]

The loss of acid phosphatase activity from human leukocytes when these cells phagocytize particles has been reported by several investigators;[40,59] their results correspond

to the findings of the present experiments and clearly differ from results of earlier studies with rabbit neutrophils, in which no loss of granular enzymes were found after phagocytosis.[42] Even with rabbit neutrophils, however, several more recent studies have demonstrated a release of granule associated enzymes comparable to the results presented here with human leukocytes.[47,50]

A net loss of enzyme activity (incubation media enzyme + cellular enzyme) noted in these experiments, when leukocytes were challenged with a high multiplicity of bacteria to be ingested, has not been reported by other investigators. Despite trying different methods of releasing the lysozyme activity from the various fractions (eg. sonication, increased freeze-thawing), I was unable to alter this result.

III. THE EFFECTS OF COLCHICINE, CORTISOL, AND SALICYLATE UPON THE EXTRA-CELLULAR RELEASE OF LYSOZYME FROM LEUKOCYTES

It has been proposed that various anti-inflammatory drugs work in part by virtue of an effect upon the mobilization of the granular enzymes contained in neutrophils. Evidence for this have been provided by a variety of studies which have demonstrated that divers anti-inflammatory drugs protect *in vitro* suspensions of lysosomes (derived from rat liver cells) against spontaneous or induced disruption which would otherwise release the activity of their bound enzymes.[33,41] (reviews) Glucocorticoids,[60,61] colchicine,[62] and acetylsalicylic acid[63] have each been reported as having a "stabilizing" effect upon rat liver cell lysosomes, although certain investigators have refuted these findings with cochicine[40,41] and salicylate.[64] Colchicine has, however, been shown to inhibit the degranulation of the intact, phagocytizing neutrophil, and it has been suggested that this drug inhibits intracellular mechanisms by which the granules fuse with phagocytic vacuoles containing ingested particles.[40,65]

The proposals that these anti-inflammatory drugs have a pharmacological action by virtue of an effect upon the release of granular enzymes within the phagocytizing leukocyte, have assumed that these digestive enzymes are also released in active form, to some extent, outside the cells and thereby become available for tissue damage at the site of neutrophil infiltration. The experiments with lysozyme presented above, and recent

studies by others with lysozyme and other granule associated enzymes, provide direct evidence that granular enzymes do escape from neutrophils, particularly when these cells phagocytize large numbers of particles.

The following experiments demonstrate that while colchicine and cortisol inhibit the release of lysozyme from phagocytizing leukocytes, salicylate does not have this effect.

Methods

Phagocytosis by Drug Treated Leukocytes:

For each experiment sufficient donor blood was drawn in order to provide leukocyte isolates (as described) for 16 leukocyte cultures. In the experiments each culture flask contained $2.9 - 3.1 \times 10^7$ leukocytes in serum buffer media as described; each flask was preincubated with or without drug for 30 min after which the leukocyte cultures were incubated with or without heat-killed Staphylococci (at adjusted concentrations such that the multiplicity of bacteria to neutrophils was 30 to 1) for an additional 60 min; each culture contained a final volume of 3.0 ml The following experimental alternatives were prepared and incubated concurrently and in duplicate:

1. Leukocytes + serum-buffer media
2. Leukocytes + media + bacteria
3. Leukocytes + media with Colchicine* (2.5×10^{-5}M)
4. Leukocytes + media with Colchicine + bacteria
5. Leukocytes + media with Na Salicylate** (2.2×10^{-3}M)

*Colchicine: U.S.P., Sigma Chem. Corp., St. Louis, Mo.

**Na Salicylate: U.S.P., Mallinckrodt Chem., St. Louis, Mo.

6. Leukocytes + media with Na Salicylate + bacteria
7. Leukocytes + media with Cortisol* ($5x10^{-4}$M)
8. Leukocytes + media with Cortisol + bacteria

In each experiment the various cultures were incubated at the same time, with leukocytes prepared from the same blood donor (although different donors were used from experiment to experiment) in order that the comparison among drug and control groups would be the more meaningful by adjusting for variations in the experimental system, eg. numbers of leukocytes, multiplicity of bacteria (an effort was made to keep these numbers as constant as possible), and different blood donors.

After incubation, cells were separated from their media, and the the cellular and media lysozyme activity was measured as previously described. None of the drugs at the concentrations used had a demonstrable effect upon the assay for lysozyme.

Several separate experiments were done in which control cultures were compared with cultures to which acetylsalicylic acid** (10^{-3}M) was added, both groups incubated as described above, with and without bacteria.

Separate experiments were also done in which isolated human neutrophil granules (prepared as described for the "granular fraction") suspended in 3.0 ml K-R phosphate buffer, with or without 10^{-3}M acetylsalicylic acid, were incubated at 37.5°C for varying periods of time (0 min, 60 min, and 180 min). After incubation

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*Cortisol: Hydrocortisone Sodium Succunate (Solu-Cortef), Upjohn, Kalamazoo, Mich.

**Acetylsalicylic Acid: U.S.P. Merck and Co., West Point, Pa.

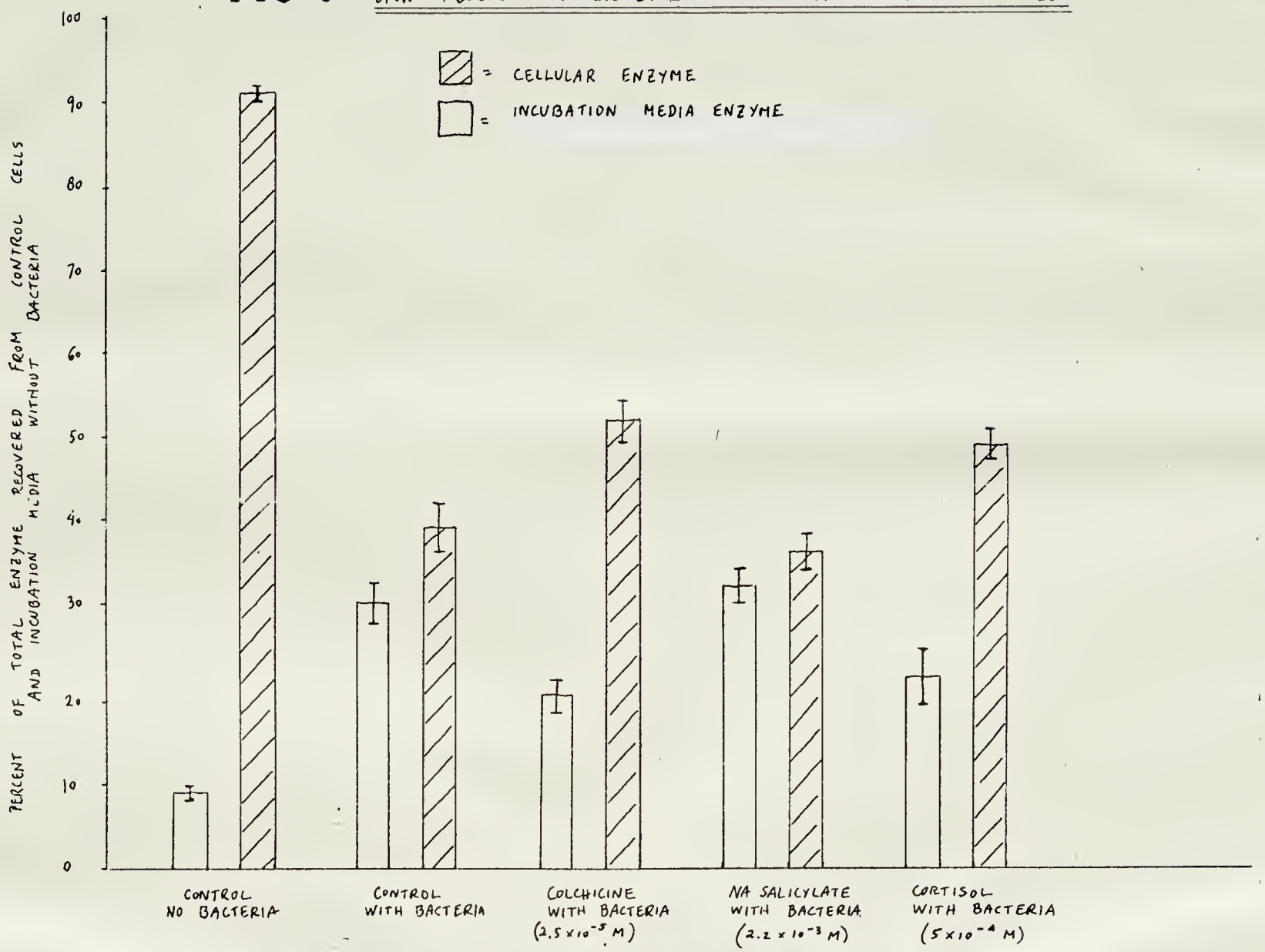
the granule suspensions were centrifuged at 10,000 rpm for 20 min (Sorvall centrifuge). The supernatants and the pellets, which were resuspended in an equal volume (up to 3.0 ml) in 0.2 M sucrose and were then freeze-thawed x 5, were both measured for lysozyme activity. (Note: The concentration of acetylsalicylic acid (10^{-3}M or 18 mg%) used in these separate experiments, lower than that used for na salicylate, was necessitated by the relative insolubility of ASA.)

Results

There was some variation from experiment to experiment in the percentage of total enzyme activity lost from the leukocytes during phagocytosis (32 - 60%) and in the percentage of total enzyme activity gained in the extra-cellular media (15 - 33%). However, within individual experiments, colchicine and cortisol inhibited the release of lysozyme from the leukocytes into the media, whereas salicylate did not have this effect. These experiments are summarized in Fig.5; since the drug treated cultures without bacteria showed no significant differences from the control cultures without bacteria, these values are not included in Fig.5. The mean differences between the control and drug treated experimental groups are given in Table 1; the differences between control extra-cellular media and the media from drug treated cultures, as well as the differences between control cell lysozyme and drug treated cell lysozyme, are statistically significant* for the colchicine and cortisol groups.

*by paired t-test

FIG. 5 EFFECTS OF COLCHICINE, NA SALICYLATE, AND CORTISOL UPON RELEASE OF LYSOZYME FROM PHAGOCYTIZING LEUKOCYTES



Total Lysozyme = 3.4 - 6.0 ugm/ml (human Lysozyme) or 11.2 - 19.8 ugm/ml (egg-white Lysozyme); results represent mean values for 10 experiments; SEM's are indicated.

pre-incubation with drugs = 30 min ; incubation with bacteria = 60 min.
multiplicity = 30 bacteria to 1 neutrophil

TABLE 1. Differences in Lysozyme Distribution between Drug Treated and Control Cells

	Colchicine	Na Salicylate	Cortisol
Mean Differences ± SEM Cellular Enzyme after Phagocytosis	13%(±1.6)*	-3.5%(±2.2)	11.2%(±1.9)
	p < .0001	p > .10, NS	p < .0002
	(10 experiments)		
Mean Differences ± SEM Medium Enzyme after Phagocytosis	-10%(±2.2)	2.0%(±1.7)	-7.3%(±1.7)
	p < .0005	p > .10, NS	p < .002
	(10 experiments)		

*Values expressed in % of total lysozyme recovered from media and cell fractions

Slides were prepared for morphology from all leukocyte cultures after incubation; all drug treated samples showed active phagocytosis by neutrophils which had been exposed to bacteria. Photomicrographs of representative neutrophils containing ingested Staphylococci are presented in Fig. 6. None of the drugs appeared to prevent or inhibit phagocytosis at the concentrations of drugs used. In Fig. 2 there is further evidence that differences in ingestion did not produce the differences observed in enzyme activity release; for this to be the case, drug treated cells would have had to have ingested only one half as many bacteria as control cells -- clearly not the case.

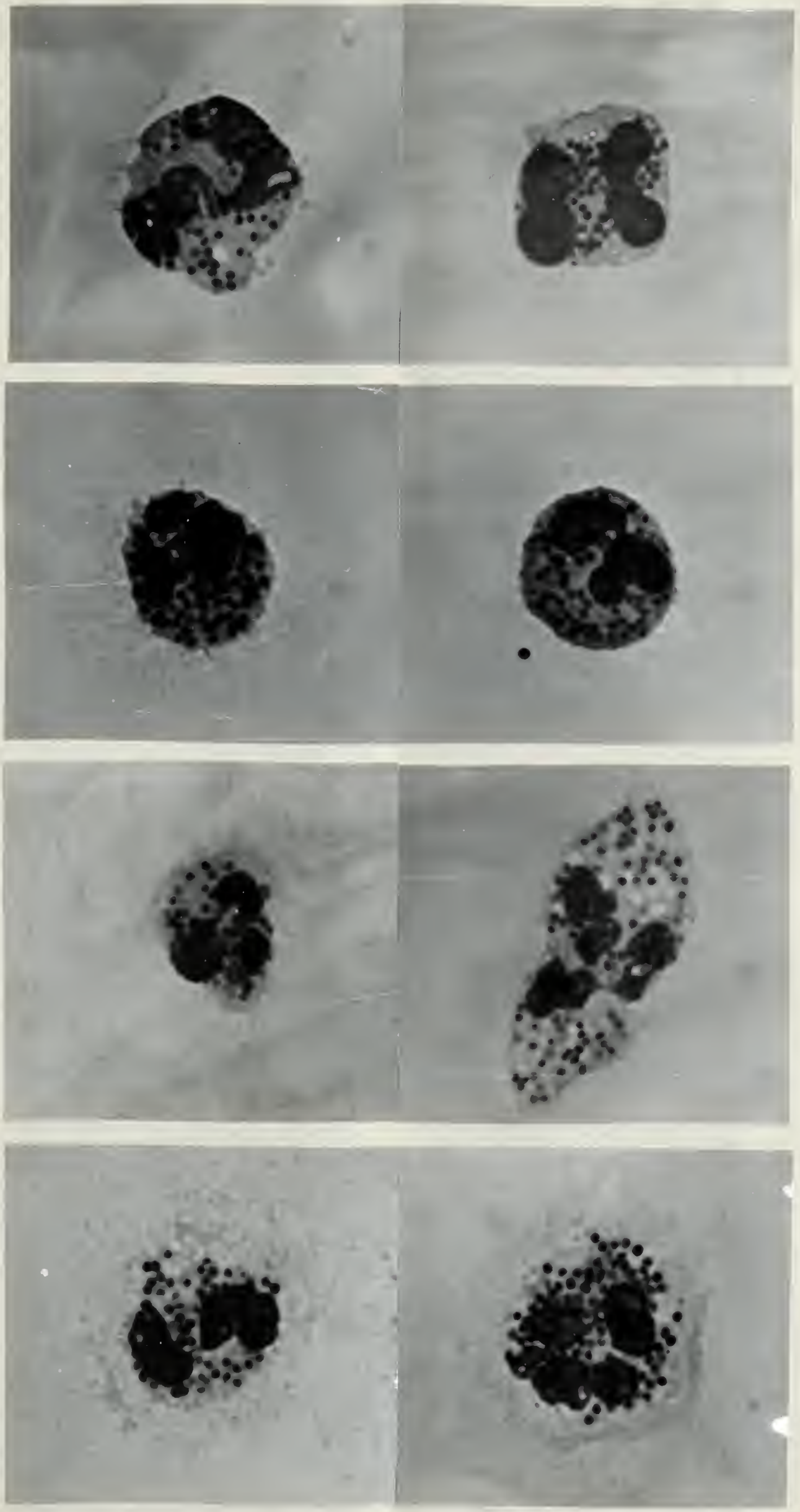
Separate experiments comparing control cells with cells treated with acetylsalicylic acid (10^{-3} or 18 mg%) failed to show an effect by this drug upon the release of lysozyme during phagocytosis (Fig. 7). In two experiments this drug also failed to "stabilize" isolated granules against spontaneous disruption when granule suspensions were incubated in the presence of the drug. (Fig. 8).

Discussion

Although both colchicine and cortisol prevent the

FIG. 6

NEUTROPHILS WITH PHAGOCYTIZED STAPYLOCOCCI



Control Cells

Colchicine Treated Cells

Salicylate Treated Cells

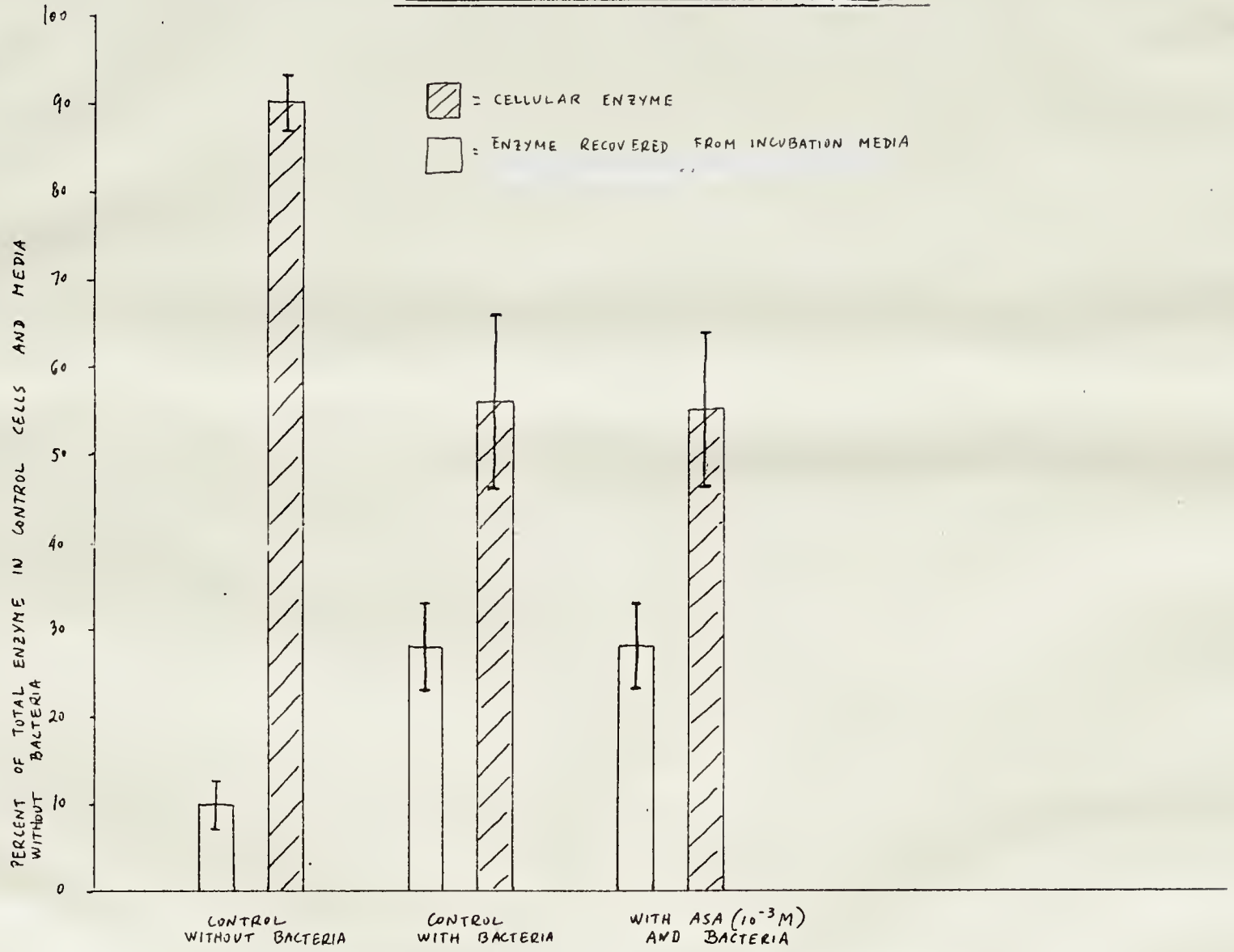
Cortisol Treated Cells

(22 mm = 10 μ)

10 μ

FIG. 7

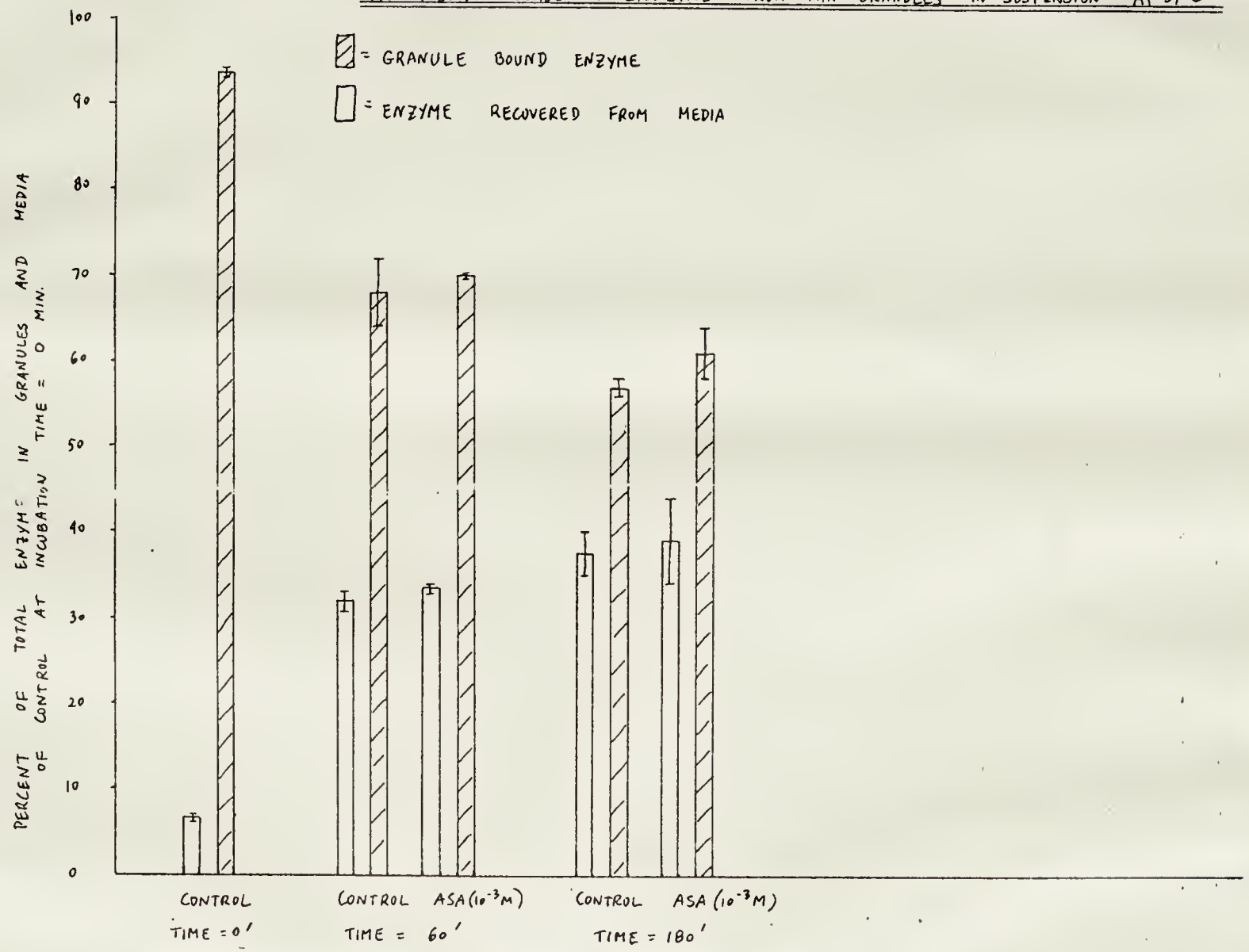
EFFECT OF ACETYLSALICYLIC ACID (10^{-3} M) UPON RELEASE OF LYSOZYME FROM PHAGOCYTIZING LEUKOCYTES



Total Lysozyme = 4.4 - 7.1 ugm/ml (human lysozyme) or 14.5 - 23.2 ugm/ml (egg-white lysozyme); results represent mean values for 4 experiments, SEM's are indicated.

pre-incubation with drug = 30 min; incubation with bacteria = 60 min; multiplicity = 30-10 bacteria to 1 neutrophil

FIG. 8 EFFECT OF ACETYLSALICYLIC ACID (10^{-3}) ON SPONTANEOUS RELEASE OF LYSOZYME FROM PMN GRANULES IN SUSPENSION AT 37°C



Total Lysozyme = 5.0 - 5.2 ugm/ml (human Lysozyme) or 16.0 - 17.2 ugm/ml (egg-white Lysozyme); results represent mean values of 2 experiments, with the ranges of values indicated.

release of lysozyme from phagocytizing leukocytes, as one would predict from the demonstrated effects of these drugs in other systems (inhibition of degranulation or "stabilization" of isolated lysosomes against disruption), salicylate was not found fo have this effect. It may be that the concentrations of salicylate used, or the time of pre-incubation of the leukocytes with drug before they were challenged with bacteria, were inappropriate for producing an effect in this experimental system. However, the concentrations of salicylate used, 1-2.2 x 10^{-3}M, represent the upper limit of a pharmacologic plasma level (18 - 35 mg%) and were larger than concentrations of salicylate previously reported to have an effect of "stabilizing" lysosomes of rat liver cells (concentrations as low as 10^{-5}M acetylsalicylic acid were reported to have this effect).[63] In several experiments in which I attempted to "stabilize" neutrophil granules against disruption, no effect of salicylate was found. Experiments presented here, in sum, refute the proposal that salicylate owes part of its anti-inflammatory action to an effect upon the mobilization of granular enzymes in inflammatory leukocytes.

On the other hand these experiments lend further support for the hypothesis that granular enzymes are mobilized by neutrophils during phagocytosis and are released in part into the extra-cellular spaces, particularly with active phagocytosis. Inhibition of this process by colchicine and cortisol may account, in part, for their anti-inflammatory actions in vivo.

It is important to note that the concentrations of

colchicine used in these experiments (and in similar experiments reported by others), are much higher than plasma levels of drug that are apparently achieved *in vivo* with normal therapeutic doses (as much as 1000 times as high by one report).[66] Because of this discrepancy between *in vitro* and *in vivo* drug levels, it has been suggested that among the numerous effects of colchicine upon divers stages of the inflammatory process only those effects which can be demonstrated at concentrations of drug *in vitro* comparable to measured plasma levels may be considered to be important effects pharmacologically.* [66] Inhibition of neutrophil chemotaxis is one effect reported to occur at such concentrations of drug *in vitro*.

Certainly this is an important consideration. However, it would be erroneous to assume that the concentrations of drug to which neutrophils are exposed in these experiments may be equated entirely or even in part to *in vivo* plasma levels; plasma levels by themselves do not indicate intracellular concentrations of drug, nor do they account for time of exposure. In these experiments drug effects are demonstrated after only 60 - 90 min exposure of neutrophils to drug, whereas the anti-inflammatory effects of colchicine *in vivo* are observed and measured over much longer periods of time. Furthermore, since colchicine appears to have a series of effects upon neutrophils,[65] it is to be expected that much lower concentrations may be re-

*Plasma levels of colchicine after therapeutic doses have been reported to range between 1 and 3 ugms/100 ml; drug concentration used in these experiments is 10ugm/ml.

quired to show an anti-inflammatory effect *in vivo* than are required to demonstrate a single effect in an *in vitro* experimental model.

A similar discussion is appropriate concerning the concentrations of cortisol used in these experiments. In studies which measured plasma cortisol levels achieved in normal volunteers after oral doses of 200 mg Hydrocortisone Acetate, maximum levels of 125 ugm/ml were found.[67] This compares with a concentration of 18 mg/100 ml of cortisol used in these experiments -- a concentration about 150 times as high as the *in vivo* plasma levels. Since therapeutic doses as high as 400 mg of oral hydrocortisone are sometimes used to suppress inflammatory diseases, and since cortisol levels achieved in inflammed tissues are known to be higher than in normal tissues,[68] one may argue that the *in vitro* levels of drug used in these experiments are more in the range of 50 times as high as the reported therapeutic plasma levels of cortisol *in vivo*. However, as with colchicine, it would be erroneous to equate measured cortisol plasma levels with concentrations of drug found to have an effect *in vitro*, for there are obvious differences in exposure times, cells to drug. Also, as mentioned before, plasma levels *in vivo*, by themselves, do not indicate cellular concentrations of drug. Furthermore, glucocorticoids, like colchicine, have been shown to have effects upon several stages of the inflammatory process,[8] and, as mentioned before, it would not be unexpected that one effect *in vitro* would require a larger concentration of drug than would the sum of several effects *in vivo*.

IV. THE EFFECTS OF COLCHICINE, CORTISOL, AND SALICYLATE UPON THE MOBILIZATION AND RELEASE OF LEUKOCYTE ACID PHOSPHATASE AND CATHEPSIN

As has been discussed, it would be dangerous to assume that all enzymes bound in neutrophil granules behave as does lysozyme. Therefore, two other granular enzymes -- acid phosphatase and cathepsin (acid protease) -- were studied in the same experimental system that has been presented. Both of these enzymes, as studied by others, have been reported to be released from neutrophils during phagocytosis.[47,48,49]

Methods

The glassware, solutions, buffer, isolation and incubation of leukocytes with and without bacteria, with and without drugs, as well as the preparation of cellular and extracellular media fractions for enzyme determinations, were as previously described.

Measurement of Acid Phosphatase:

An assay for acid phaosphatase which uses a-naphthyl acid phosphate as a substrate was chosen for several reasons.[69] First, since I wished to measure enzyme activity in an incubation media made up largely of phosphate buffer, it was necessary to use an assay which did not depend upon the measurement of free phosphate split off from a substrate. Second, this assay proved itself to be, as others found it, very sensitive[70] and reproducible. Third, in rabbit neutrophils there are at least two acid phospha-

tases with different substrate specificities: the assay that uses p-nitrophenyl phosphate as a substrate measures enzyme activity which for the most part is not located in the granular fractions as are other lysosomal enzymes, whereas an assay using B-glycerophosphate as substrate measures enzyme activity located almost exclusively in the small neutrophil granules.[57,58] The assay chosen for these experiments, a fluorometric method, has been observed to have the same substrate specificity as the latter substrate and to be more sensitive.[70]

0.1 ml of sample to be measured was mixed with 1.0 ml of 0.1 M acetate buffer at pH 6.1 and with 1.0 ml buffer which contained a concentration of 5 mM a-naphthyl phosphate sodium* (or 60 mg/50 ml). Each individual sample was measured in duplicate, together with a blank, consisting of 0.1 ml sample and 2.0 ml buffer; sample blanks were measured in each case in order to correct for background fluorescence of the sample itself. The acetate buffer was made up before the assay procedures and stored at 4°C; the substrate-buffer solution was prepared fresh with each set of assay measurements. The sample-substrate-buffer mixtures were incubated at 37°C in a shaking water bath for 15 min, after which 1.0 ml of 0.5 N NaOH was added and the mixture immediately measured for fluorescence in a FOCI Spectrophotometer Mark I, Ferrand Optical Co.** Acid phosphatase

*Mann Research Labs, N.Y.,N.Y.

**Incident wave length, 340 mu, emitted wave length, 455 mu, optimal for measuring fluorescence of free a-naphthol which is the reaction product promoted by enzyme activity.

activity is expressed as uM/ml free a-naphthol/ 15 min, corrected for sample blank measurements. Colchicine and cortisol were not found to interfere with the assay; salicylate did, however, cause an interference which is discussed below.

Measurement of Cathepsin:

The assay for cathepsin used in these studies has been used commonly in studies of leukocyte granular enzymes.[27] It measures proteolysis of denatured hemoglobin at pH 3.7.[71] 0.5 ml of sample to be measured was added to 0.5 ml distilled water and 1.0 ml substrate solution.* Each sample was measured in duplicate. Control blanks of 1.0 ml distilled water and 1.0 ml substrate solution were also prepared. Samples with serum-buffer alone or with each of the three drugs in the experimental concentrations were also measured in order to adjust for serum enzyme or for drug interference. As with the assay for acid phosphatase, only salicylate was found to interfere with the assay (discussed below). All sample-substrate mixtures were incubated in a water bath at 37°C for 2 hrs, after which 2.0 ml of a 5% TCA solution was added to stop the reaction, precipitating undigested hemoglobin protein. The assay mixtures were incubated at 37°C for an additional 1 hr and were then centrifuged at 1500 rpm (International centrifuge) for 10 min. The

*2.2% denatured hemoglobin (Hemoglobin Substrate, Mann Research Labs., N.Y.) in 0.05 M acetate buffer at pH 3.7

supernatants were pipetted into new tubes and stored over night at 4°C. The supernatants were then measured for free peptides by the Lowry method[72]: 0.4 ml of supernatant sample was mixed with 2.0 ml "reagent I"* and allowed to stand for 10 min, then 0.2 ml "reagent II"** was added and mixed rapidly. After the mixtures had been allowed to stand for an additional 30 min, they were read in a spectrophotometer*** at 750 mu for optical density. Results were translated into ugm protein/ml/ 2 hrs. from a standard curve prepared concurrently from protein solutions (bovine albumin) of known concentration. Results were corrected for assay blank values.

Effects of the Drugs upon the Enzyme Assays:

None of the drugs affected the total cellular enzyme activities recovered from control leukocytes without bacteria. Neither colchicine nor cortisol affected the assays for enzyme activities in the incubation media. Salicylate, however, interfered with both assays for incubation media enzyme activities. Salicylate had an intrinsic fluorescence at the wave lengths used in the acid phosphatase assay, but it was possible to adjust for this extra fluorescence by subtracting sample blank values (described above). Similarly, salicylate interfered with the cathepsin assay by causing a color reaction by itself in the

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*100 parts 3% sodium carbonate in 0.1 N NaOH, 1 part 4% sodium potassium tartrate, 1 part 2% copper sulphate.

**a 1:2 dilution of 2 N Phenol reagent, Fischer Scientific.

***Hitachi Perkin-Elmer #139

Lowry reagents -- this interference was adjusted for by subtracting salicylate-media blank measurements from sample measurements.

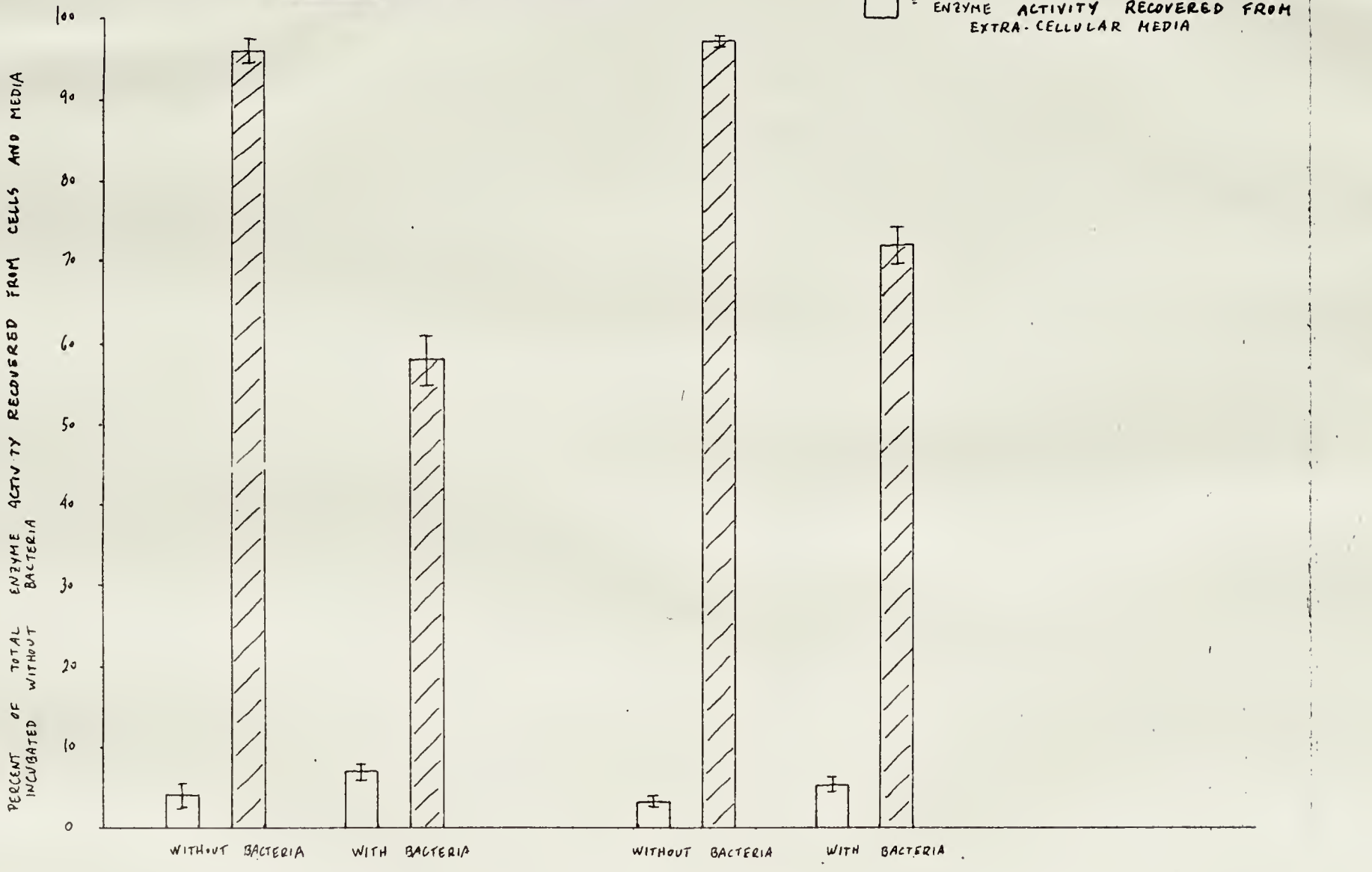
Results

As was demonstrated with lysozyme, phagocytosis of bacteria by neutrophils is accompanied by a loss of acid phosphatase and cathepsin activity from the cells and an increase in the activities of these enzymes recovered from the incubation media (Fig. 9). However, the percentages of total enzyme activities recovered from the extra-cellular media fractions are much smaller than those observed in measurements of lysozyme, and the differences of these enzyme activities in the incubation media of phagocytizing leukocytes from the enzyme activities in the media of non-phagocytizing cells were much too small to demonstrate significant drug effects. On the other hand differences among enzyme activities recovered from the cell fractions were quite sufficient to demonstrate significant drug effects (Fig. 10 and Table 2). As in experiments with lysozyme, colchicine and cortisol consistently inhibited the loss of enzyme activities from the leukocytes during phagocytosis, whereas salicylate did not have this effect. The differences between the experimental groups and the controls were consistently reproduced from experiment to experiment and are statistically significant.*

It was thought possible that smaller percentages of acid phosphatase and cathepsin were recovered in the extra-cellular incubation media, than percentages of lysozyme, because of

*by paired t-test

FIG. 9 RELEASE OF ACID PHOSPHATASE AND CATHEPSIN FROM PHAGOCYTIZING LEUKOCYTES



ACID PHOSPHATASE

TOTAL ACID PHOSPHATASE ACTIVITY = $1.0 - 2.2 \times 10^{-2}$ μM α-NAPHTHOL / 15 MIN

RESULTS REPRESENT MEAN VALUES FOR 8 EXPERIMENTS
SEM'S ARE INDICATED

CATHEPSIN

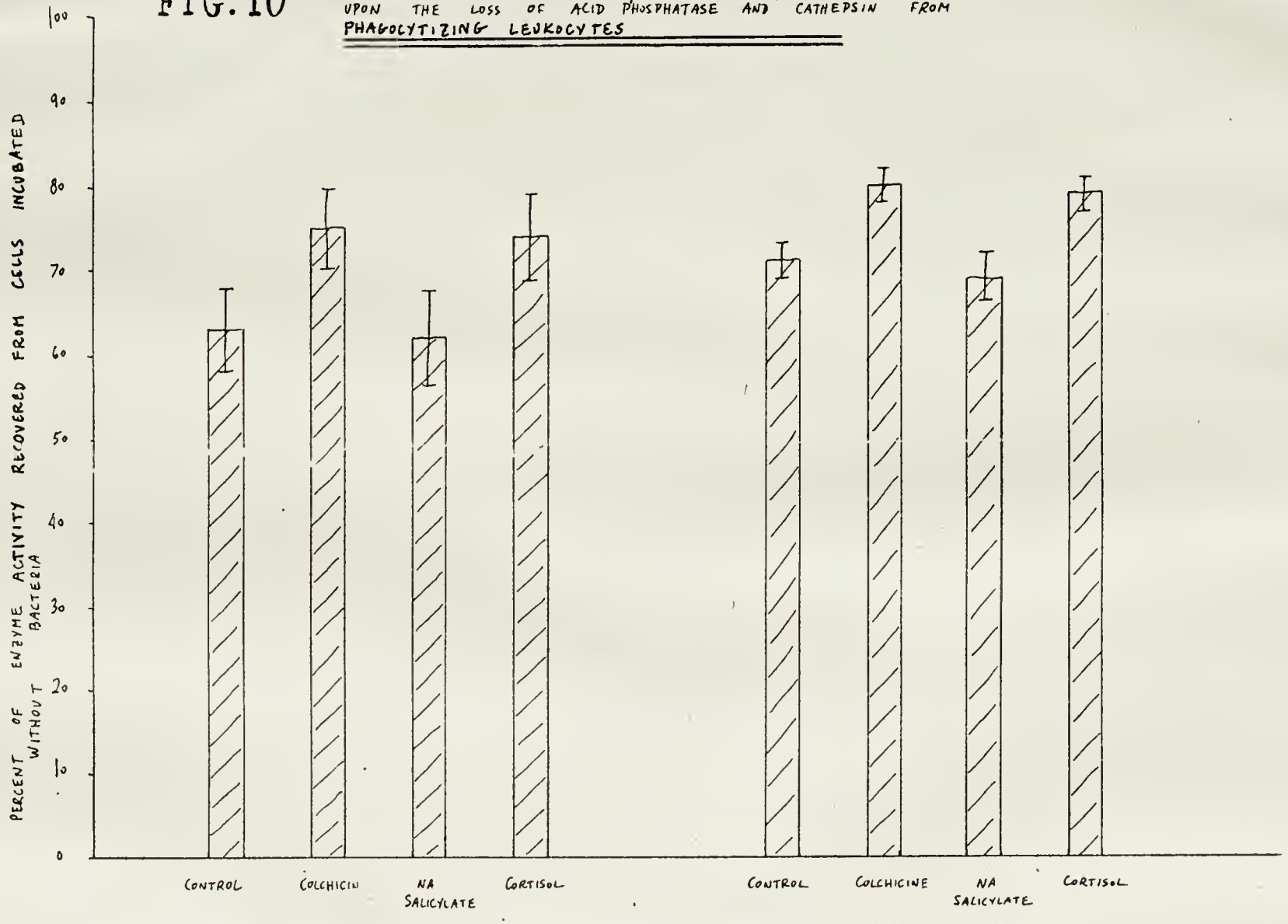
TOTAL CATHEPSIN ACTIVITY = 139 - 239 μgm/ML PROTEIN/2 HRS.

RESULTS REPRESENT MEAN VALUES FOR 8 EXPERIMENTS
SEM'S ARE INDICATED

total incubation time = 90 min ; incubation with bacteria = 60 min. ; multiplicity = 30 bacteria to 1 neutrophil

FIG. 10

THE EFFECTS OF COLCHICINE, NA SALICYLATE, AND CORTISOL UPON THE LOSS OF ACID PHOSPHATASE AND CATHEPSIN FROM PHAGOCYTIZING LEUKOCYTES



ENZYME ACTIVITY RECOVERED FROM CELLS WITH BACTERIA

ACID PHOSPHATASE

TOTAL ACID PHOSPHATASE ACTIVITY = $.9 - 1.9 \times 10^{-2}$ μM α-NAPHTHOL / 15 MIN

MEAN VALUES FOR 6 EXPERIMENTS WITH SEM's INDICATED

ENZYME ACTIVITY RECOVERED FROM CELLS WITH BACTERIA

CATHEPSIN

TOTAL CATHEPSIN = 136 - 233 μgm PROTEIN / ML / 2 HRS.

MEAN VALUES FOR 6 EXPERIMENTS WITH SEM's INDICATED

pre-incubation with drugs = 30 min.; incubation with bacteria = 60 min; multiplicity = 30 bacteria to 1 neutrophil

TABLE 2. Differences in Cellular Acid Phosphatase and Cathepsin between Control and Drug Treated Cells

	Colchicine	Na Salicylate	Cortisol
Mean Differences ± SEM, Cellular Acid Phosphatase after Phagocytosis	13.8%(±2.4)* $p < .001$ n = 8	-1.3%(±1.4) $p > .20$, NS n = 7	13.0%(±2.9) $p < .005$ n = 6
Mean Differences ± SEM, Cellular Cathepsin after Phagocytosis	7.1%(±1.4) $p < .001$ n = 8	-1.9%(±1.4) $p > .10$, NS n = 7	8.3%(±2.0) $p < .005$ n = 6

*Values expressed in % of total enzyme recovered from non-phagocytizing control cells

differences in the stabilities of these enzymes in the experimental system. To test this hypothesis, two supplementary experiments were done. In the first of these experiments leukocyte lysates were incubated for varying periods of time in the serum-buffer media. Leukocyte suspensions were freeze-thawed in the presence of 0.1% Triton-X 100 to lyse the cells and release the granular enzymes into solution; aliquots of lysate in serum-buffer media were then measured immediately for enzyme activity or were incubated for 30 to 60 min to see if enzyme activities decreased during this time of incubation at 37.5°C. Representative results from these experiments are indicated in the left-hand half of Fig. 11. Acid phosphatase and cathepsin clearly demonstrate different stability characteristics than does lysozyme, the first two enzymes losing 50 - 60% of their activity in 60 min, while lysozyme loses no more than 8% of its activity during this time.

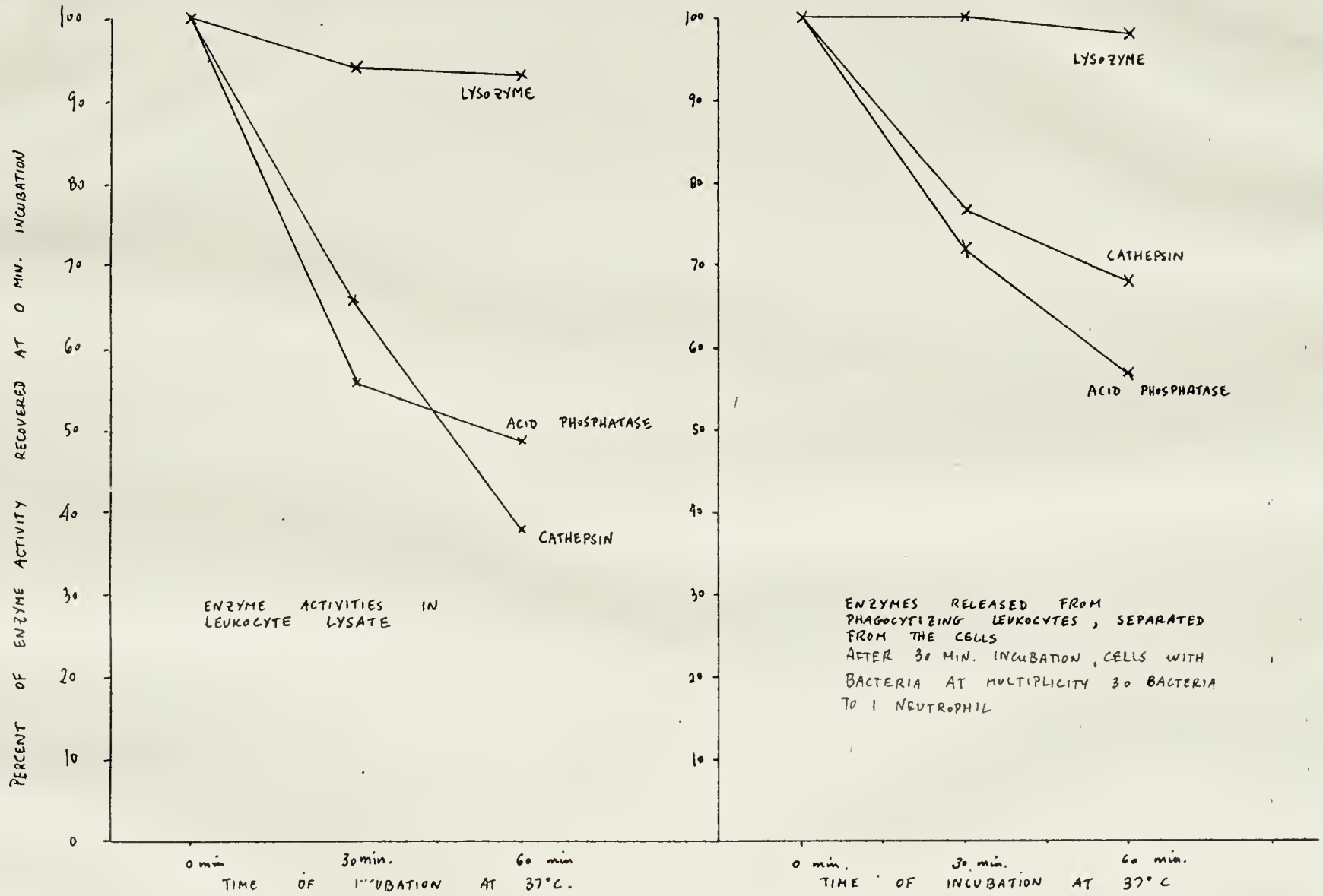
Since this experiment compared the behavior of enzymes contained in a crude cell soup and not of enzymes which had been

released exra-cellularly by intact leukocytes, according to the process described by previous experiments, a second group of supplementary experiments was done. Since it was known that some increased acid phosphatase and cathepsin activity was recovered from the extra-cellular media of phagocytizing leukocytes, (although this increased activity was small) large numbers of leukocytes ($3\text{-}5\times10^7$/ml) were incubated with bacteria at a multiplicity of about 30:1 for 30 min (sufficient time for most of the enzyme release to occur according to Fig. 3), with the intention of causing a large release of enzyme during phagocytosis. After the incubation, the cells were separated from the media by centrifugation, and aliquots of media were either measured immediately for enzyme activity or were incubated for varying additional periods of time before enzyme activities were measured. Representative results are presented in the right-hand half of Fig. 11. The lysozyme released extra-cellularly from leukocytes loses little activity with further incubation, whereas the acid phosphatase and cathepsin released from the cells are clearly unstable.

Discussion

As is the case with lysozyme, human leukocytes also lose acid phosphatase and cathepsin, other granule associated enzymes, during phagocytosis. In addition, as was shown with lysozyme, colchicine and cortisol inhibit the loss of these granular enzymes from the leukocytes, while salicylate fails to have this

FIG. 11 RELATIVE STABILITY OF LYSOZYME, CATHEPSIN AND ACID PHOSPHATASE IN THE INCUBATION MEDIA



effect. The inhibition by colchicine of the loss of acid phosphatase from phagocytizing leukocytes confirms previously published data, in which a different assay for acid phosphatase was used.[40] Measurements of enzyme activities in the extra-cellular incubation media did not reproduce the results obtained when lysozyme was measured; although consistent increases in acid phosphatase and cathepsin activities were observed with phagocytosis, these increases were small and differences were too small to demonstrate significant drug effects. These two enzymes, however, when incubated free in culture media, proved to be much less stable than lysozyme, and it seems likely that acid phosphatase and cathepsin are released from the phagocytizing neutrophils as is lysozyme, but are not recoverable in convincing amounts because of their relative instability during the 60 min period of incubation. It is also possible, of course, that some of these enzymes are degraded within the cells and are not released extra-cellularly.

The question of enzyme stabilities has not been reported as important in other studies of this kind. In the present studies, however, phagocytosis clearly is accompanied by decreases in total measureable enzyme for all three granular enzymes investigated. In one reported study, which compared phagocytosis by normal human leukocytes with phagocytosis by leukocytes from patients with Chronic Granulomatous Disease, a decrease in total acid phosphatase activity recoverable after phagocytosis

was observed with the normal cells; furthermore, this decrease in total enzyme activity was not reproducible with rabbit neutrophils. These investigators, however, did not attempt to explain their observations by studying enzyme stabilities in their experimental system.[59]

V. THE EFFECTS OF COLCHICINE, CORTISOL, AND SALICYATE UPON THE EXTRA-CELLULAR RELEASE OF LEUKOCYTE "NEUTRAL" PHOSPHATASE

Because it was apparent that the release of acid phosphatase from phagocytizing leukocytes may be masked by the instability of this enzyme when freed into the medium, several experiemnts were done in which phosphatase substrate was added to the incubation media, By this maneuver, enzyme released from the leukocytes could act upon the substrate immediately, thus "trapping" the enzyme activity before degradation of the enzyme could occur. For these experiments it was assumed that the phosphatase-associated reaction occurred at pH 7.4, the pH of the serum-buffer media; therefore, the enzyme activity measured in these experiments is referred to as "neutral" phosphatase. The effects of colchicine, cortisol, and salicylate were compared under these new experimental conditions.

Methods

The preparation and incubation of leukocytes with bacteria was as previously described. Drug-treated leukocyte cultures were also prepared as described; however, a-naphthyl phosphate substrate was added to the serum-buffer media such that the leukocytes were incubated with substrate at a concentration of 1.75 mg/ml. It was calculated that this addition ofsubstrate (a sodium salt) added approximately 28 mOsm to the osmolarity of the incubation media (from ~308 to 336 mOsm). The incubation of leukocytes with enzyme substrate is not without precedent;

a study of the 1950's used an experimental system whereby leukocytes were incubated in the presence of lysozyme substrate in order to quantify leukocyte damage secondary to various insults (eg. hypotonicity of the extra-cellular medium, endotoxins) -- lysis of the substrate was taken as evidence of leukocyte damage with extrusion of leukocyte enzymes.[73]

After the leukocyte cultures were incubated with or without bacteria, with or without drug, the cells were removed by centrifugation. The extra-cellular media fractions were then made strongly alkaline with the addition of 2.0 ml of 0.5 N NaOH (necessary for fluorescence of free a-naphthol) and were immediately measured in a spectrofluorometer (as described above). Measurements were adjusted for "blank" values, obtained by measuring the background fluorescence of serum-buffer-substrate media, with and without the three drugs, which had been incubated without leukocytes -- it will be remembered that salicylate was found to cause a sizeable background fluorescence at the wavelengths used for measurements. The fluorescence measurements obtained from the media of non-phagocytizing cells were compared with measurements from media containing phagocytizing cells. Slide preparations of leukocytes showed that substrate did not prevent phagocytosis.

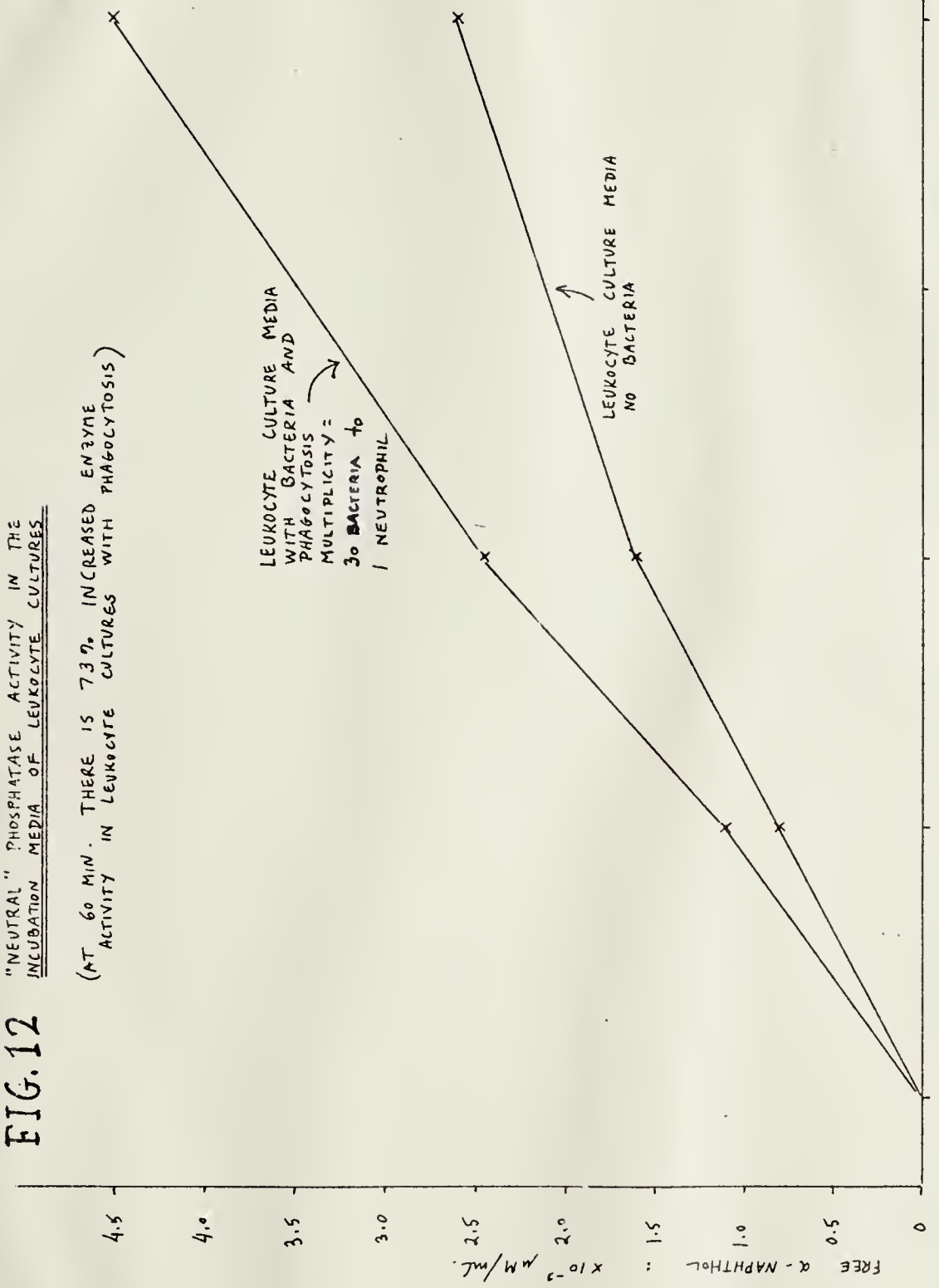
Results and Discussion

Fig. 12 illustrates the increase of fluorescence (or free a-naphthol) in the extra-cellular media of phagocytizing

FIG. 12

"NEUTRAL" PHOSPHATASE ACTIVITY IN THE INCUBATION MEDIA OF LEUKOCYTE CULTURES

(AT 60 MIN. THERE IS 73% INCREASED ENZYME ACTIVITY IN LEUKOCYTE CULTURES WITH PHAGOCYTOSIS)



RESULTS REPRESENT MEAN VALUES OF THREE EXPERIMENTS ADJUSTED FOR FLUORESCENCE IN SERUM-BUFFER-MEDIA "BLANKS"

and non-phagocytizing leukocyte cultures, indicating a rise in extra-cellular phosphatase activity with ingestion of bacteria by neutrophils. Table 3 represents values for the percentages of increased phosphatase activity in the culture media with phagocytosis for control and drug treated groups after incubation periods of 1 hr.* The increases in extra-cellular phosphatase activity with phagocytosis is less for colchicine and cortisol treated cultures than for controls, whereas the percentage of increased enzyme activity is slightly more for the salicylate-treated cultures. The values given in Table 3 represent 6 experiments; variabilities were large and the means are statistically different at relatively high "p" values. However, the differences among the drug-treated and control groups are consistent with the results of experiments presented above.

Table 3. Percentages of Increased Phosphatase Activity* in the Incubation Media with Phagocytosis

	Control	Colchicine	Na Salicylate	Cortisol
Mean values of Percentages Increased Enzyme Activity ±SEM	62%(±18.4)	26%(±5.6)	73%(±18.6)	30%(±10.2)
		$0.02 < p < 0.05$	$p < 0.05$	$p < 0.02$

p values calculated from paired t-test

It is important to note that "neutral" phosphatase activity (activity at pH 7.4) recovered from the leukocytes was found to be located almost entirely in the "granular" fraction, when cellular fractions were separated as previously described. It must be added that, although these results are compatible with

*these percentages are comparable to the figure of 73% noted in Fig. 12, which is the percent increased phosphatase activity found in the media of phagocytizing cells compared with phosphatase activity found in the media of non-phagocytizing cells (at 60 min.)

findings already presented concerning the actions of the three drugs studied, these experiments do not exclusively record extra-cellular enzyme activity. Phosphatase substrate was found to enter the leukocytes during phagocytosis (not unexpectedly) and to be broken down within the cells, as was evidenced by the appearance of fluorescence within the leukocytes (fluormetric measurements were made on lysates of cells which had been separated from the media at various incubation times). At least a part of the increased phosphatase activity found in the incubation media with phagocytosis may represent free a-naphthol which had been split from the substrate within the cells and then leaked out into the media.* Certainly, these experiments demonstrate that colchicine and cortisol inhibit the intracellular mobilization of granule-associated enzyme activity; however, these experiments also provide evidence that these drugs inhibit the extra-cellular release of granular enzymes during phagocytosis.

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*As is apparent in Fig. 12 there is phosphatase activity in the incubation media of non-phagocytizing cells, evidenced by rising concentrations of free a-naphthol during incubation; part of this rise represents phosphatase activity of the serum in the media; part of this rise represents instability of the a-naphthyl phosphate substrate; however, some of this rise also represents a reaction of the substrate with the non-phagocytizing cells, for which I have no explanation.

VI. SUMMARY AND CONCLUSIONS

The role of the Polymorphonuclear Leukocyte (Neutrophil) in natural protection against infectious disease has been recognized since the turn of the century. Now, this cell is also recognized to be of great importance as a mediator of tissue damage in a variety of acute inflammatory diseases in which the inflammatory stimuli are harmless without the inflammatory response which they provoke. The pathologic role of the neutrophil has been delineated best in such experimental models of human disease as provided by the Arthus Phenomenon and crystal-induced acute arthritis, in which an inflammatory response and not stimulus is destructive to the animal or human host.

In particular, the various digestive enzymes and specific proteins bound in the granules of neutrophils have been proposed as the active agents contributed by neutrophils in mediating the tissue damage of acute inflammation. It is known that these granular enzymes and proteins are mobilized from their inactive, bound state during phagocytosis for purposes of destroying ingested particles. Also, for some time it has been assumed that in acute inflammation these lytic agents become available in the extra-cellular spaces and cause tissue damage, and recently in various in vitro experimental systems it has been demonstrated directly or indirectly that granular substances are released from neutrophils during phagocytosis. Such findings in vitro

are compatible with several studies *in vivo* in which increased plasma or transudate fluid levels of neutrophil granular enzymes have been found coincident with natural or experimental acute inflammation; in particular, lysozyme has been found to be increased in various body fluids with acute inflammation[74] -- in fluids from acute arthritic joints,[75,76] and in sera of rabbits injected with large numbers of bacteria or concentrated suspensions of antigen-antibody complexes.[50]

In this report *in vitro* studies have been presented which quantitate in detail the loss of lysozyme from human leukocytes during phagocytosis together with the appearance of lysozyme in the extra-cellular incubation media. These studies have also quantitated the release of lysozyme during phagocytosis as a function of the time of phagocytosis and as a function of the phagocytic challenge. Kinetic studies of the release of lysozyme have shown that the release of the enzyme occurs during phagocytosis, not at a time when phagocytosis has been completed. These studies have also indicated that the release of lysozyme appears to approach a maximum at a certain high level of phagocytic challenge. Furthermore, these studies indicate that the release of lysozyme represents a process which is selective for granule-associated substances and does not represent destruction of the leukocytes; lactic dehydrogenase, a non-granular, cytoplasmic enzyme, was not found to be released during phagocytosis together with lysozyme.

Other studies also presented demonstrate that two

anti-inflammatory drugs, cochicine and cortisol, inhibit the phagocytic release of lysozyme. A third anti-inflammatory drug, salicylate, did not affect the release of lysozyme, and the failure of salicylate to affect this process of granular enzyme release is taken as strong evidence that this drug does not act upon this stage of the inflammatory process as has been previously suggested.

Studies with acid phosphatase and cathepsin, neutrophil enzymes which are also bound to the granules of these cells and are also mobilized during phagocytosis, have demonstrated that these enzymes are lost from phagocytizing leukocytes as is lysozyme. Similarly the cellular loss of these enzymes during phagocytosis has been shown to be inhibited by colchicine and cortisol but not by salicylate. Obvious increases in acid phosphatase and cathepsin activity extra-cellularly were not found as a consequence of phagocytosis, in contrast to findings with lysozyme; however, further studies which demonstrated the relative instability of these two enzymes in solution offer a likely explanation for this finding.

Studies, in which leukocytes phagocytized bacteria in the presence of phosphatase substrate, confirmed the findings of lysozyme experiments that granular enzymes appear in active form extra-cellularly during phagocytosis and that this process is inhibited selectively by colchicine and cortisol but not by salicylate.

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Although, as Metchnikoff suggested, disruption of neutrophils may be an important mechanism in the release of lytic substances from neutrophils in acute inflammation, it is apparent that granular enzymes may be released from neutrophils during active phagocytosis without destruction of these cells. Recently it has been suggested that the release of granular enzymes from phagocytizing neutrophils may occur only with the ingestion of certain kinds of particles (eg. "undigestible particles").[77] However, the fact that release of granular enzymes has been demonstrated or suggested in studies with phagocytosis of a great variety of particles makes this proposition unlikely. The kinetic studies presented in this report relate the release of granular enzymes directly to the process of phagocytosis and not to an effect of the ingested particle upon the leukocyte once inside the cell. It was once thought that virus particles (or other particles of small size) were phagocytized by neutrophils but did not cause mobilization of granular enzymes within these cells; recent quantitative studies, however, have shown that as soon as viruses (opsonized by specific antibodies) are cleared from solution by neutrophils *in vitro*, the usual intracellular events of phagocytosis, including loss of granular enzymes, occur -- when leukocytes are incubated with viruses but do not undergo these intracellular changes, they also fail to remove the viruses from solution.[78]

This same point has been made in an *in vivo* model: when sodium urate microcrystals of varying sizes are infused

into joint spaces, it appears that only crystals large enough to be ingested by neutrophils provoke an inflammatory response.[15]

The observation that different neutrophil granular enzymes are, to varying degrees, unstable in solution would appear to be an important one. These instabilities most likely account for the decreased total enzyme activities found in leukocyte cultures in which phagocytosis has occurred. The instabilities of granular enzymes may reflect an intracellular mobilization of enzyme inhibitors; in any case it is not unexpected that the potential dangers of the toxic granular substances might be naturally limited by short half-lives of enzymes once released into solution. One may postulate that the partial extrusion of granule-associated lytic enzymes from neutrophils during phagocytosis of invading micro-organisms is generally of no consequence to the host tissues because of the short half-lives of these enzymes when free in solution. The release of granular enzymes becomes of consequence in producing acute inflammation, however, with circumstances in which there exists sudden, localized, and intense phagocytosis.

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